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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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The number of newly engineered nanomaterials is vastly increasing along with their applications. Despite the fact that there is a lot of interest and effort is being put into the development of nano-based biomedical applications, the level of translational clinical output remains limited due to uncertainty in the toxicological profiles of the nanoparticles (NPs). As NPs 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. The initial screening for nanotoxicity is preferably performed *in vitro*, but extrapolation to the *in vivo* outcome remains very challenging. In addition, generated *in vitro* and *in vivo* data are often conflicting, which consolidates the *in vitro–in vivo* gap and impedes the formulation of unambiguous conclusions on NP toxicity. Consequently, more consistent and relevant *in vitro* and *in vivo* data need to be acquired in order to bridge this gap. This is in turn in conflict with the efforts 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 and routine methods for nanotoxicity evaluation, and give an overview of adjustments that can be made to the cultivation systems in order to optimise current *in vitro* models. We will also 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 large number of applications since the 1990's. Today, inorganic nanoparticles (NPs) are applied in various technological applications and consumer goods. For example, zinc oxide (ZnO) NPs are used in sunscreens and toothpastes and silver (Ag) NPs can be found in food packages and deodorants and are applied as a preservative in cosmetics.[1–3] Given the ever increasing use of NPs and the high interest in exploiting the exceptional features of NPs in biomedical applications, it is expected that both intentional and unintentional exposure will become more frequent.[4,5] Consequently, the increasing implementation of nanotechnology in our daily lives is joined with raising concerns on potential adverse effects on human health.[6] It is therefore recommended that the safety of these products, for consumers and workers at the production site, is carefully evaluated before their introduction into 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] This legislation should cover all aspects of nanotechnology without any material or application being left out, which is, from a practical point of view, extremely hard to obtain. In order to try and overcome this predicament, the European Commission has launched a recommendation on a definition of nanomaterials in 2011 that states that a nanomaterial is: ''a natural, incidental or manufactured material containing particles, in an unbound state or as an aggregate or

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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 on the correct definition, but the most used criterion is the size limitation.[9–11] Nanotechnology is subsequently defined as the manipulation and application of particles and systems with at least one dimension below 100 nm.[10] For nanomedicine purposes these technologies are used to develop applications for diagnosis,[12,13] imaging,[14,15] treatment[16,17] and prevention of diseases.[18–20] A recent novelty in nanomedicine is the concept of theranostics, where particles combine diagnostic and therapeutic features in a single construct.[21,22] For example, 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]

It is on the one hand due to the minute dimensions of the NPs that they exhibit many unique properties (*e.g.* IONPs are superparamagnetic[24] and gold (Au) NPs have a localised surface plasmon resonance[25]) because of which they can be implemented in novel innovative applications.[26] These dimensions are on the other hand often the cause of adverse health effects through the higher surface area to volume ratio and enhanced surface reactivity.[27,28] The fact that both the great potential and the high risk lie in the miniature dimensions of the NP is referred 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.[29] Nanotoxicology is referred to as the study on interactions between NPs and biological systems with an emphasis on

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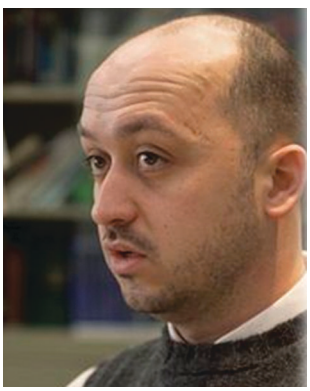

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establishing a relationship, if any, between the physicochemical properties of the NPs and the toxicological responses.[30] It is crucial that nanotoxicology is regarded as a distinct category of toxicology since standard toxicity assays, initially developed for the evaluation of chemical substances, are often inadequate for nanotoxicity assessment. This can be attributed to the different mechanisms leading to nanotoxicity, the specific behaviour of the NPs in culture media and the possible interference of NPs with various toxicity assays.[9,31–33] Therefore the classical toxicity testing paradigm needs to be optimised to be applicable for nanosafety evaluation. The experimental design is, besides the assays, also subject to optimisation, as it is clear from the literature that it has the potential to influence the uptake and/or the observed toxicological effects.[34–37]

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 in order to minimize artefacts and resemble the *in vivo* situation more closely, which will be illustrated with recent findings from the literature.

2 Nanotoxicology

2.1 Common mechanisms of nanotoxicity

As mentioned above, nanotoxicology should be regarded as a specific subcategory of toxicology since the general toxicology paradigm cannot completely cover the toxicity induced by NPs.[29] Whereas most chemicals induce cell damage through interactions with specific biomolecules, 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.[30,38,39]

Generally higher levels of toxicity are observed for NPs in comparison to the bulk material.[20,30] Important factors in this respect are the higher surface area to volume ratio, higher surface reactivity and susceptibility to degradation and ion leaching.[27,40] Furthermore, most NPs are unstable in dispersion, as they are prone to agglomeration or aggregation and sedimentation, which influences NP uptake and toxicity.[34,38,41]

Nel *et al.* have put ROS induction forth as one of the main mechanisms through which inorganic NPs induce toxicity since this effect has been observed in a multitude of *in vivo* and *in vitro* studies.[42–46] For instance, Wang *et al.* observed a significant decrease in the GSH/GSSH ratio in the olfactory bulb and hippocampus in mice after intranasal exposure to IONPs.[47] 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 24 h IONP exposure.[48] ROS can be induced by several mechanisms, including (i) the interference with redox active proteins, (ii) the 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) the activation of several signalling pathways through interaction with cell surface receptors.[27,38] This paradigm does however not account for all NPs: cerium oxide (CeO_2) NPs, for example, were found not to induce ROS but on the contrary even showed a protective effect against ROS damage *in vivo* as well as *in vitro*.[49,50] A comparable effect was shown for IONPs, as they exhibited an intrinsic peroxidase-like activity in a cell free environment as well as in mesenchymal stem cells, where they even promoted cell proliferation.[51,52] Unlike for ROS induction the mechanisms causing this protective effect have not yet been unravelled. However, it was shown that only unimpaired IONPs displayed a peroxidise-like activity, whereas upon degradation of the IONPs, considerable ROS levels were noticed. The effects are therefore likely to depend on the coating, as it influences NP uptake levels, intracellular localization and resistance against pH-dependent degradation. Together, these findings certainly

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underscore the complexity of nanotoxicology and the importance of obtaining a toxicological profile that covers as many facets as possible.

Persistent ROS induction at high levels, leading to oxidative stress, may have very grave consequences since it can cause many secondary effects such as protein denaturation, modulation of specific signal transduction pathways, inflammation, (mitochondrial) membrane damage and DNA damage.[27,53] However, DNA damage through ROS induction is not the only route to genotoxicity, as it is known that NPs can also alter gene expression *via* interactions with signal transduction pathways or the transcriptional or translational machinery through perinuclear localisation.[54,55] Finally, very small NPs with a diameter below 5 nm may directly interact with DNA.[56] ROS can furthermore cause actin stress fibre formation and therefore alter the cell's morphology, motility and adhesion.[57,58] In turn morphological changes may cause mitochondrial membrane depolarization, which leads to higher ROS levels.[59]

Secondly, NPs may be susceptible to degradation in a physiological environment when they are exposed to an oxidative environment in late endosomes or lysosomes after endocytotic uptake. Here they face degrading enzymes like cathepsin L and a pH of 5.9 in late endosomes or 4.5 in lysosomes.[38,60] This can cause degradation or dissolution of the NPs, resulting in the leaching of free ions or an increase in reactive surface groups.[38] The following impact on cell wellbeing depends on the chemical composition of the NPs. For Cd-containing quantum dots (QDs), for example, the leaching of highly toxic Cd^{2+}-ions is considered to be the main cause of any observed toxicity.[61,62]

Finally, it is known that NPs avidly bind serum proteins to their surface, creating a protein corona.[63] The nature of this corona depends on the NPs physicochemical properties and the composition of the microenvironment (*e.g.* cell culture media) surrounding the NPs.[30,64] The binding of serum proteins to the NP surface is an important determinant in how the cells 'see' the NP and therefore influences NP uptake and toxicity.[65–67] Additionally, proteins incorporated in this corona can undergo conformational changes, because of which the cell may recognize them as an antigen and initiate an immune response.[20,29] The protein corona can also stimulate opsonisation by macrophages *in vivo*, thereby activating the complement system and evoking an immune reaction.[68] A final pathway to immunotoxicity is the enhancement of an allergic immune response.[69,70]

In the current nanotoxicity paradigm, ROS induction has thus been set forth as the main toxic effect, 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 be addressed when evaluating nanotoxicity, implying the need to evaluate a number of parameters *via* a multiparametric method.

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*, with the former typically preceding any *in vivo* work.[71,72] It is essential to note that, prior to any toxicity testing, the NPs must be thoroughly characterized with respect to the purity (chemical and biological contaminants) and physicochemical properties in both a dry and wet state.[72,73] More detailed information on NP characterisation will not be given as this falls beyond the scope of the current review, but can be found in overviews elsewhere.[73–76] This section summarizes the main methodological principles for *in vitro* and *in vivo* nanotoxicity evaluation as well as their most important shortcomings.

2.2.1 Routine *in vitro* methods. *In vitro* assays are mainly the first to be conducted during a toxicological evaluation.[77] 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 generally depends on the expected *in vivo* target organ and application of the NPs.[78] In a vast majority of the studies the cells are exposed to the NP dispersion during a single incubation period, which mostly ranges from 3 up to 48 hours.[77,79,80] The induced toxicity is subsequently evaluated using mainly biochemical assays, including enzymatic and (enzyme-linked) immunoassays with a fluorometric or spectrophotometric read-out due to the relatively short duration of most assays, the uncomplicated detection principle and straightforward data processing.[81] Furthermore, the possibility of upscaling and automation of the execution, detection and data processing makes these assays highly convenient in regard to a future high content screening approach.[81,82] Another popular method is to stain the entire cell or specific cellular components with cellular dyes, fluorescently labeled antibodies (AB) or molecular probes that interact with a specific biomolecule.[38,77,83] This approach allows detection *via* a plate reader or flow cytometry but is to the utmost extent combined with microscopy-based analysis. The latter is an important tool for the evaluation of morphological features like cell spreading, but will also increasingly be applied in high content screening approaches to evaluate cellular processes.[84,85]

Finally specialized techniques are used to evaluate specific parameters. For example, ion leaching can be detected in cell-free conditions using specialized buffers and the intactness of stem cell functionality can be evaluated by observing the efficiency of cellular differentiation induced by specific protocols.[38,86]

2.2.2 Routine *in vivo* methods. Subsequently *in vivo* studies are performed to test a dose range derived from *in vitro* experiments or realistic exposure doses.[87] Most studies, certainly preliminary studies, are conducted on rodents as costs are lower, animals easier to access and infrastructural requirements less elaborate.[71] In order to reduce the number of animals used for toxicity testing, as urged by the 3R concept by Russel and Burch, ethical criticism and the pressure to develop a more cost-effective toxicity assessment approach, official instances have determined the species and number of animals that should be used for a specific test.[88,89] The European Commission has bundled its guidelines in the REACH (Registration, Evaluation and Administration of Chemicals) regulation. This regulation was plainly adopted to nanotoxicity

Table 1 Assays performed in an *in vitro* and *in vivo* setting

	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	[a]
Immunotoxicity and sensitization		X
Genotoxicity	X	X
Carcinogenicity		X
Reproductive toxicity		X
Toxicokinetics		X

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

evaluation even though it was initially designed for chemical substances.[90] However, this approach has been put to question since classical toxicity assays show major shortcomings when they are applied for nanotoxicity assessment (as will be discussed in Section 2.2.3). For assay protocols REACH 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'.[89]

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 NPs toxicokinetics.[89] (A comparison of the parameters that are evaluated in an *in vitro* or *in vivo* setting is given in Table 1.) The REACH regulation furthermore postulates the data that need to be collected and the endpoints for different aspects of the toxicity evaluation. For instance, when performing carcinogenicity experiments the endpoint is the appearance of tumours. These are subsequently resected and analysed in terms of number, size, shape *etc.*[91] For most aspects 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.[89] Probably the most used technique for *in vivo* studies is the histopathological examination of selected organs and tissues from a sacrificed animal since this method is used in all required aspects except for the skin and eye irritation or corrosion and skin sensitisation and genotoxicity experiments.[89,92–94] Examination of the organs furthermore includes the evaluation of the morphology (in terms of length, width and shape) and tissue colour.[89] Besides analysis of whole organs, any assay performed in an *in vitro* setting can be performed on cells obtained from the (sacrificed) animal. This way, more detailed information can be obtained on, for instance, *in vivo* ROS induction, inflammation or activation of the immune system. These *in vivo* effects can subsequently be clarified by the molecular mechanism found in *in vitro* experiments.

2.2.3 Issues with routine *in vitro* and *in vivo* methods. Up to now, many conflicting data have been generated as can be found in reviews listing data for the toxicity of a specific NP or the correlation between the physicochemical properties of NPs and the toxic effects they evoke.[28,95–97] This led to an increasing awareness that the routine methods, and especially the *in vitro* methods, may not be as appropriate for nanotoxicology purposes as was previously assumed.[87,88] Nel *et al.* 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.[27] We believe that the major issues with the current *in vitro* methods are (i) the (mostly) incomplete NP characterisation, (ii) the lack of consensus on the dose metric, (iii) the lack of standardisation and guidelines on how to perform an *in vitro* toxicological evaluation, (iv) the possibility of NP interfering with the assays and (v) the shortcomings inherent to the most used classical 2D monocultures.[11,87,88,98,99] Therefore, it is 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.

NP characterisation. A first shortcoming to the current nanotoxicity testing strategy for *in vitro* and *in vivo* studies is the often incomplete characterisation of the particles. Currently, it is nearly impossible to retrieve reliable conclusions on the effect of a specific parameter since many physicochemical parameters such as NP size, charge, shape *etc.* often differ between studies. This originates from the fact that altering one parameter, for example surface charge, without affecting any other (hydrodynamic size, colloidal stability, nature of the coating...) is not an easy task.[99] Comparing effects found in different studies is furthermore impeded by the fact that all essential data on physicochemical characterisation may not always be provided. Therefore consensus should be reached on which parameters must be characterised as well as on the methods applied for this purpose. We believe that information should at least be provided on the following parameters: NP composition (core and coating) and purity, size, charge, surface area and agglomeration.

NP concentration. Besides the NPs 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.[100,101] Expressing the concentration in terms of mass per volume is the easiest option but not always the most relevant, as smaller NPs often evoke a stronger toxic response in comparison to their larger counterparts at similar mass per volume doses.[37,99] Wittmaack considers particle number per volume to be the best dose metric.[102] Along with other groups, we believe that the concentration should be expressed in terms of surface area per volume since both particle size and number are contained in this metric and it is known that (1) toxicological responses depend on the surface properties of the NPs and (2) that the surface area exponentially increases with a decrease in NP size. Furthermore, this metric is not influenced by differences in particle density, which is the case for the mass per volume metric.

However, if authors prefer to use the NP mass per volume ratio to express the concentration, it should be combined with the NP number per volume concentration to provide sufficient information in order to enable interpretation of the toxicity data between different studies.[43,100,103] The applicability of the surface area per volume unit has been demonstrated by Rushton *et al.* who found a significant correlation between the *in vitro* oxidative response and the inflammatory response *in vivo* for a group of nine different NPs, with distinct physicochemical properties, when the concentration was expressed in surface area per volume and the steepest slope method was applied.[104] When the same group applied this method to results from 2007, in which no *in vitro–in vivo* correlation could be observed, a clear correlation was now established.[104,105]

Standardisation of toxicology methods. Subsequently attention should be paid to standardisation of nanotoxicity methods in terms of incubation conditions such as the NP dose range and incubation time.[106] The setup of a relevant dose range that mimics actual human exposure to NPs is a major issue for *in vivo* nanotoxicity testing.[87] 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 gastro-intestinal tract and the skin being the main portals of entry.[11,72,107] Additionally, exposure may occur during every stage of the NPs lifecycle, be it the development, manufacture, use and disposal of the NPs.[108] Thus, the extent and complexity of this problem require 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 that enable measuring exposure doses in a standardised manner in different settings.

Incubation conditions for *in vitro* experiments also require standardization, as overexposure levels should be avoided. The importance thereof becomes clear when evaluating genotoxicity for example, since acute toxicity at overexposure conditions can mistakenly be interpreted for genotoxicity, as apoptosis is associated with DNA fragmentation.[107,109] Therefore, genotoxicity should be evaluated at sublethal doses. However, the determination of relevant dose ranges is severely hampered by the lack of exposure data and doses required for specific applications. Therefore, *in vitro* (and *in vivo*) toxicity testing currently focuses on the determination of the No Observed Adverse Effect Level (NOAEL) values instead of evaluating realistic exposure scenarios or dosages.[37]

Another important factor inducing variation in nanotoxicity data is the medium in which the NPs are dispersed. The medium can affect the agglomeration or aggregation state of the NPs, which in turn determines its behaviour in dispersion and subsequently the uptake and toxicity.[110,111] Therefore some groups have focussed on the development of methods to create a stable and uniform dispersion *via* surface modification, addition of surfactants *etc.*[112–114] However, results from a study by Oberdorster *et al.*, using surfactant stabilised dispersions, have been put to question since the observed toxicity might have been caused by surfactant residuals.[115] This leads to the assumption that it is better not to alter the dispersion state before adding the dispersion to the cell culture but to strive for a medium resembling the *in vivo* environment as close as possible.[108,116]

Sample preparation for *in vivo* studies is also subject to optimisation. Issues in this regard are the lack of a consensus on whether agglomerates should be redispersed prior to administration and the hydrophobic properties of most NPs, causing especially the preparation of reproducible aerosols to be very challenging.[83,126]

Assay interference. A fourth major issue is the potential of NPs to interfere with assays in various ways.[87] NPs with optical properties can, for instance, alter the outcome of an assay based on a spectrophotometric or fluorometric read-out.[32] Additionally, NPs may interact with enzymes or substrates because of their high absorbance capacity and/or catalytic activity.[87,98] Kroll *et al.* looked into the interference of 24 well-characterised NPs with four frequently used *in vitro* assays and observed concentration, NP and assay-dependent interferences.[31] Consequently it is imperative to validate the assays for each specific type of NPs by assuring that the measured toxicity, or the lack thereof, is indeed caused by the NPs and is not merely a consequence of interference with the assay.[117,118] Therefore appropriate controls should be introduced: besides a negative (no treatment) and positive (maximum effect) control, the positive control should be tested in combination with the NPs. Test reagents should also be incubated with the NPs to rule out any possible interaction with its components.[119] Furthermore, every single parameter should be evaluated with multiple assays, which ideally supply complementary information and have a different assay- and detection principle, to validate the obtained results.[120]

NPs do not appear to influence the outcome of *in vivo* experiments as gravely since there are, to our knowledge, currently no reports on this phenomenon. However, scepticism has been raised on the subject, as interactions may not have been uncovered yet due to the relatively low number of *in vivo* studies in comparison to the vast amount of *in vitro* data.

Finally, cellular uptake levels, intracellular localization, *in vivo* biodistribution and clearance profiles depend on various detection strategies that are correlated to the type of NPs tested and may therefore differ for different types of NPs. However, adapting a NP to enable detection by a specific technique may not be the most suitable approach, as linking a fluorescent probe to the particle may influence the formation of the protein corona or the NP–biomolecule interactions and therefore alter its *in vivo* behaviour and toxicological profile.[72] Finally, methods should be developed to obtain information on the *in vivo* NP stability to ascertain whether NPs remain unimpaired or undergo degradation, as this will influence the observed toxicity and may elucidate toxicity mechanisms.[30]

Shortcomings to classical 2D monocultures. Monocultures are static models with a very limited level of complexity in contrast to the complex and dynamic *in vivo* environment and therefore

show several important shortcomings.[30,121] A first shortcoming is the loss of intercellular communication between different cell types since most *in vitro* experiments use a single cell type, mostly the parenchymal cell type from the target organ, in an attempt to predict the *in vivo* effect.[37] But as organs consist of multiple differentiated cell types, all with their specific function, modelling the *in vivo* response by only using a single cell type is nearly impossible.[122] This is believed to be a major factor contributing to the large discrepancies that are often found between *in vitro* and *in vivo* data (see Chapter 5).

Another important shortcoming is the loss of the specific 3D tissue architecture and cellular polarisation (see Chapter 6). Therefore, 2D cultures 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.[123] Besides, the extent of ECM production is altered and the barrier formed is less dense and incomplete in comparison to the one *in vivo*.[124] This ECM is a very important factor as it is a key regulator in homeostasis and phenotype expression and forms a natural barrier with small pores, limiting NP diffusion into the tissue.[125] Consequently, cells cultured in a 3D setup are able to acquire tissue-like organization and differentiation to levels that have thus far been impossible to reach in a classical 2D setting.[123]

Other important shortcomings are the cell-type dependency of the observed nanotoxicity and the sedimentation of the NPs on top of the cells, which will respectively be discussed in Chapters 3 and 4.

Conclusion. Overviewing these findings, it can be concluded that routine *in vitro* and *in vivo* methods in part need to overcome the same hurdles, namely (i) the need for detailed NP characterisation, (ii) the development of standardized sample preparation methods and toxicity assays and (iii) the possible interference with assays and detection. An additional challenge, especially for *in vivo* studies, is the setup of a relevant dose range based on the possible exposure scenarios. 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 is stimulated by the combined efforts of various research groups.

2.3 Novel methods for toxicity testing

Once shortcomings to the current methods were pointed out, several groups started working on the optimisation. Especially the optimization of *in vitro* methods receives a lot of attention since they will become the basis of a highly needed screening approach. This section provides a number of new insights and methods for nanotoxicity assessment, guided by examples from recent literature.

2.3.1 Particokinetics. NP dosimetry poses several challenges as mentioned in Section 2.2.3. Teeguarden *et al.* proposed the concept of particokinetics to model the solution dynamics of the NPs to meet this challenge.[110] They marked diffusion, sedimentation and aggregation as predominant processes determining the NPs faith in dispersion. Additionally, 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 (Fig. 1). The latter is the most interesting for nanotoxicity and cell labelling studies but is also the most difficult 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 dose combining the administrated dose and the NPs particokinetics.[110] Recently the model was optimized and is now referred to as the *In vitro* Sedimentation, Diffusion and Dosimetry model or ISDD.[126] However, some debate on the applicability of the model remains, as it has been stated that the hereby-calculated doses will be underestimated for monodisperse NP suspensions since ISDD does not take conventional forces into account that develop in most solutions.[127]

2.3.2 Multiparametric nanotoxicity evaluation. Since it was observed that NPs can cause multiple effects *via* different mechanisms, awareness has risen on the necessity to evaluate nanotoxicity *in vitro* by a multiparametric method.[39,81] 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.[120,128,129] Endpoints such as acute toxicity, ROS induction, morphological alterations, genotoxicity and NP degradation have been put forward as important parameters that should be included.[38,120,128] Even though no consensus on the optimal design of this multiparametric method has been reached yet, several examples have been

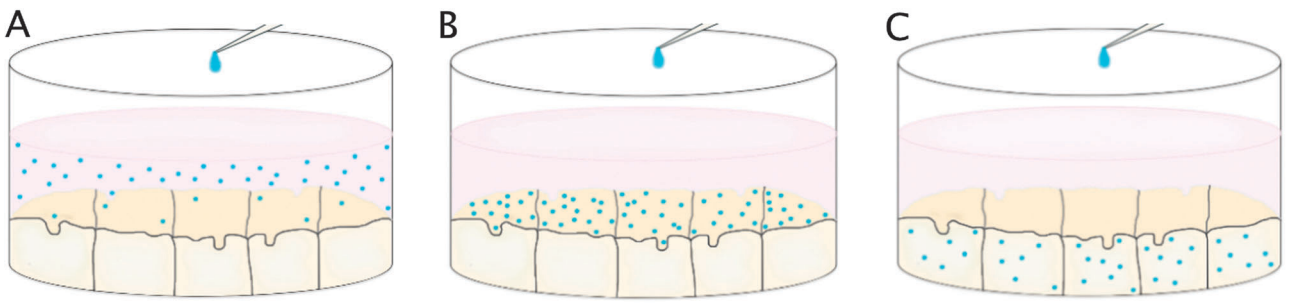

Fig. 1 Schematic representation of the concept of administered dose (A), delivered dose (B) and cellular dose (C) in terms of NP uptake and correlation NP toxicity with effective cell-associated concentrations.

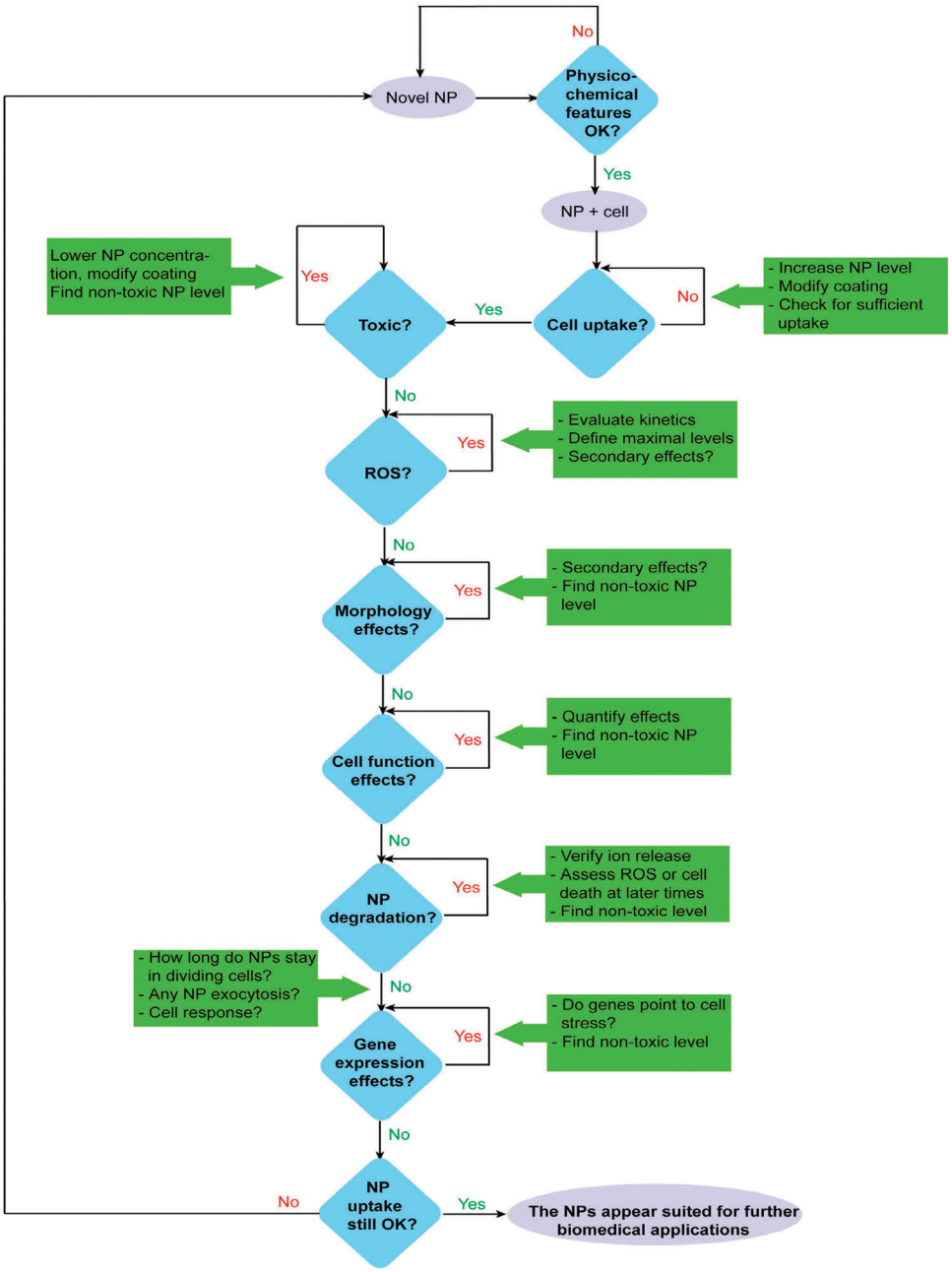

Fig. 2 Schematic overview of a multiparametric methodology that can be applied for in-depth analysis of cell–NP interactions under reproducible conditions, enabling an easy comparison of NP functionality under non-toxic conditions. This figure is reproduced with permission from Soenen *et al.*[38] © Elsevier 2011.

proposed recently, including one from our group (Fig. 2).[39,119] We believe that methods to evaluate the different parameters should preferably be biochemical or microscopy-based assays, as they are easily amendable for a future screening approach.[120] This is imperative to enable simultaneous testing of multiple doses in different cell types by using various assays in a reasonable amount of time.[120,128] Several groups also suggest the implementation of omic-techniques to screen for genotoxicity, alterations in protein expression or biomarkers related to cellular pathways.[82,130] Such a screening approach appears to be very promising since the use of a five-parameter HCS method was shown to have a significantly higher specificity and sensitivity in comparison to a single parameter approach.[131]

2.3.3 QSAR and *in silico* models. 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 the 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.[129]

A popular subject in bio-informatics is the setup 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.[129] 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 bioinformatic tools are on the other hand indispensible for an efficient processing of HCS data.[129] Recently, Puzyn *et al.* developed a QSAR that predicts the toxicity of metal oxide NPs in *E. Coli* based on the effect of 17 different metal oxide NPs.[132] However, many obstacles still need to be overcome before the implementation of the first QSAR that allows the prediction of the adverse effects of any NP towards human health. Therefore, clear correlations between the NPs physicochemical properties and observed effect must be found, large-scale comparative studies should be performed and the QSARs must be validated, which requires the identification of reference nanomaterials with well-known effects.[38,81]

2.3.4 Novel *in vivo* methods. Recently some research has been performed on the applicability of zebrafish embryos in an *in vivo* screening approach. Similarly as for most *in vitro* methods used in nanotoxicity assessment, Hill *et al.* initially developed this method for the toxicity evaluation of chemical compounds.[133] Subsequently, the suitability of this model for nanotoxicity evaluation was shown in follow-up studies examining Au NP, Ag NP and QD toxicity.[134–136] The main advantages to other *in vivo* models are the reductions in cost, labour and infrastructural requirements as zebrafish are small and have a short life cycle.[134] Another important breakthrough is the use of an integrated screening approach using the zebrafish model and HCS to obtain a hazard ranking of different NPs.[137]

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 due to their 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.[138] 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.[139,140] These 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 for *in vitro* nanotoxicity evaluation. Subsequently, the use of primary cells or stem cells has been put forward as an alternative since it is assumed that these cells mimic the *in vivo* cellular situation more closely due to the minimal *in vitro* manipulation in comparison to cell lines.[140,141] But as these cells are not always easily obtained and require specific handling, this assumption needs to be proven first by comparative studies in order to revise the current *in vitro* toxicity testing strategy. Primary cells may in turn suffer from batch-to-batch differences, which could lead to a reduced reproducibility of the experiments and is not an issue for 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.

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

Cell lines	Primary or stem cells
Readily available	Not always readily available
Less expensive	More expensive
Easy to cultivate	Require specific handling
No interbatch differences	Interbatch 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

This section summarizes several studies that verified whether various cell types differ in their way of handling administered NPs in terms of NP uptake and cytotoxicity.

3.1 Effect of the cell type on nanoparticle uptake and processing

It has been shown in many studies that most NPs enter the cell through the process of endocytosis. Some exceptions can be found as for phagocytotic cells phagocytosis remains the main uptake mechanism.[142] Wang *et al.* furthermore observed quantum dot (QD) uptake by passive diffusion in red blood cells.[143] But this must be put into perspective, as red blood cells are not capable of endocytosis.

The uptake mechanism is highly important as it determines the intracellular location of the NPs. NPs entering the cell by passive diffusion will directly interact with the cytosol, while NPs taken up by endo- or phagocytosis are retained in vesicles that are distributed widely in the cytoplasm or localized in a specific cellular region.[143,144] The uptake kinetics and intracellular location were compared in different cell types since they are important determinants for the final toxic response. For example, the uptake of 1.9 nm diameter Au NPs was found to be higher in human prostate and breast cancer cell lines (DU154 and MDA-MB231) than in a human lung epithelium long-lived cell line (L132).[145] Diaz *et al.* compared the uptake of five different NPs 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 NPs tested but mouse macrophages showed an even higher uptake, which was comparable to the uptake in PC3 cells. The monocytic cell line did not show NP internalisation, which is in conflict with results from other studies showing a higher uptake capacity for

cancer cell lines.[145–147] As all other cell types tested did not show a significant uptake the authors have put the phagocytic machinery in combination with the cell type forward as predominant factors for NP uptake.[148] This is the general belief and is supported by several studies.[149]

Regarding intracellular localization of the same particles in different cell types, widely varying results have been obtained. PEGylated micelles, for example, were shown to have similar distributions in a human lung cancer cell line and long-lived cell line (A549 and MRC-5) and a human kidney epithelium long-lived cell line (293T).[138] However, Barua and Rege observed significant variations in intracellular localisation of QDs in three phenotypically closely related human prostate cancer cell lines (Fig. 3). It was observed that the QDs 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.[150] 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.[150]

It is clear that the cell type is an important factor for NP uptake, influencing the uptake mechanism as well as the extent of NP uptake. With cancer cell lines mostly showing higher uptake levels when compared to uptake in long-lived cell lines and primary cells. Intracellular distribution also appears to be cell type dependent and has even been found to differ substantially between phenotypically closely related cell lines. Therefore these findings underscore the importance of selecting a representative cell type that mimics the *in vivo* situation as closely as possible, as toxicity is logically related to the uptake determining the cellular dose of the NPs.

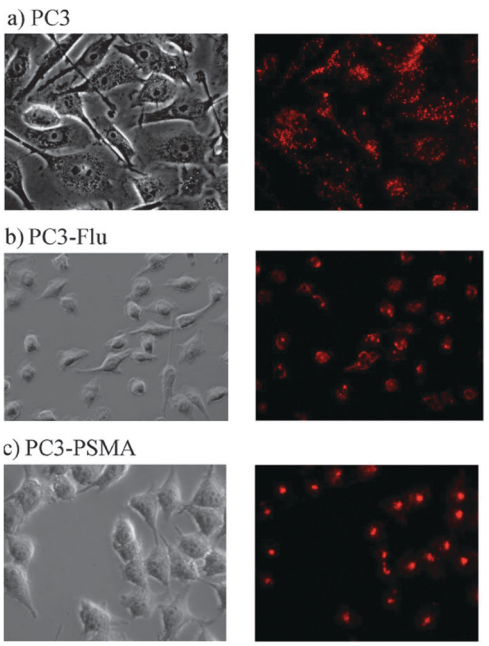

Fig. 3 The effect of cell phenotype on NP processing as illustrated by the clear differential intracellular localization patterns of QDs in highly similar, but slightly differing human PC3 cells. (a) PC3, (b) PC3-flu, (c) PC3-PSMA. This figure is reproduced with permission from Barua and Rege.[150] © Wiley-VCH 2009.

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 leading to discordant *in vitro* nanotoxicity data. Since the use of cell lines for *in vitro* nanotoxicity assessment has been put to question, several groups compared the effects of NP exposure in cell lines to those in primary or stem cells representing the same tissue. In general the primary cells and stem cells have been found to be less sensitive to NP toxicity than long-lived cancer cell lines.[139,151] For instance, Hanley *et al.* found two human T-cell lymphoma cell lines (Jurkat and Hut-78) to be respectively 28- and 35-fold more sensitive to ZnO NP exposure than human primary T-cells (Fig. 4A).[152] In contrast to these studies Bregoli *et al.* observed an impaired proliferation in primary human hematopoietic progenitor cells after Sb_2O_3 NP exposure while the proliferative capacity of none of the seven hematopoietic (cancer and long-lived) cell lines tested was affected.[140]

As the focus of *in vitro* toxicity testing is shifting to the use of primary cells, it was put to question whether cellular differentiation has an influence on the induced toxicity. Several studies show

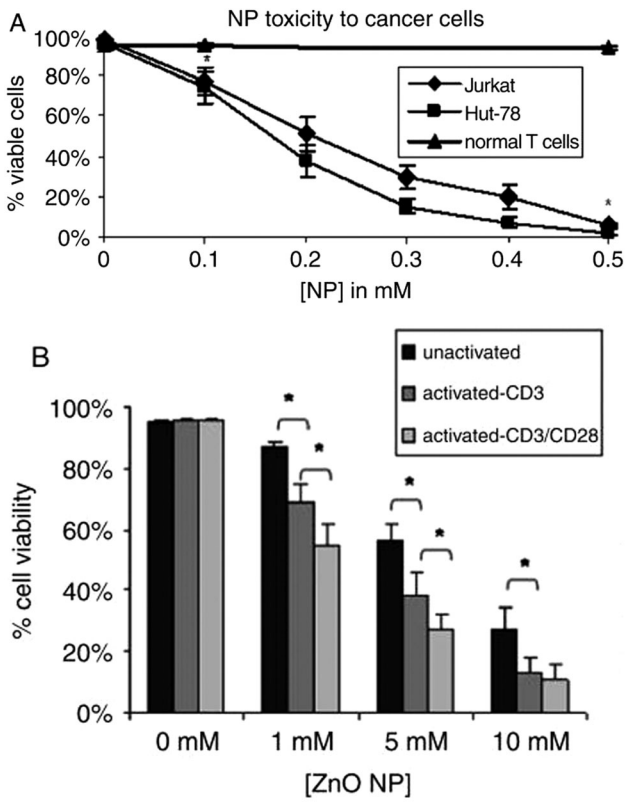

Fig. 4 The effect of cell phenotype on NP toxicity as illustrated by (A) differential viability after ZnO NP exposure of Jurkat and Hut-78 cancer cell lines and normal human T lymphocytes and (B) differential viability after ZnO NP exposure for unactivated and activated human T lymphocytes. This figure is reproduced with permission from Hanley *et al.*[152] © IOP Sciences 2008.

a higher sensitivity towards nanotoxicity for differentiated cells, which can be explained by the fact that undifferentiated cells adapt better to new conditions than differentiated cells.[153,154] Saretzki *et al.* found the stress defence mechanism (antioxidant capacity and DNA repair mechanism) in murine embryonic stem cell to be superior to that of various differentiated murine cells (fibroblasts, hematopoietic progenitor cells and 3T3 fibroblast long-lived cell line).[155]

A number of groups furthermore tried to elucidate the mechanisms causing various cell types to react differently to NP exposure. First of all the physiological cell function appears to be important. For instance, macrophages and monocytes are involved in the clearing of xenobiotics as they can ingest many compounds in high quantities by phagocytosis, which explains the higher uptake levels and susceptibility to nanotoxicity.[148,149,156] A second factor is the variation in proliferative capacity of different cell types: rapidly proliferating cells can experience less toxicity due to the fast dilution of the cellular NP contents with every cell division. This was shown by Chang *et al.* as they found three epithelial cancer cell lines (A549, MKN-28 and HT-29, average doubling time 23 h) to be less prone to silica NP induced injury than three long-lived fibroblast cell lines (WS1, CCD-966sk and MRC-5, average doubling time 128.4 h), which was explained by the higher cell division rate of the former and the associated dilution of cellular NP levels.[157] Additionally, NP uptake depends on the cell cycle phase, being maximal in G2/M phase. Therefore, rapidly proliferating cells may experience more toxicity due to higher NP uptake levels. This was demonstrated by Hanley *et al.* as activated primary human T lymphocytes showed a higher susceptibility to ZnO NP induced injury in comparison to resting human T lymphocytes due to the preferential targeting of the actively proliferating cells (Fig. 4B).[152] A final factor is the cellular natural antioxidant level since Mukherjee *et al.* accounted the higher toxicity levels found in HeLa cells compared to those in HaCaT cells (a human dermal cancer cell line) to the lower natural antioxidant capacity of the former, as Ag NP caused stronger ROS induction and GSH depletion in HeLa cells.[158] This observation is in line with ROS induction being the main common mechanism inducing nanotoxicity.[27]

It is clear from these data that the response to NP exposure is highly cell type-dependent since primary cells are mostly found to be 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, as differentiated primary cells were shown to be more prone to nanotoxicity. The observed 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 NP hazard assessment, which is a balancing act since both primary cells and cell lines show specific advantages and major shortcomings. Therefore, further research is needed in order to determine the proper cell system and optimized cell culture protocols.

4 The effect of NP agglomeration and sedimentation

As mentioned before, is *in vitro* nanotoxicity generally evaluated in a similar setup as for chemicals? Since chemical substances typically dissolve in the cell medium, is the dose to which the cells are exposed accurately represented by the concentration of the chemical in solution? In the first instance it was assumed that this also applied for NPs, as they were thought to be evenly dispersed by Brownian motion.[34] This appeared not to be true as NPs in dispersion are not only subject to diffusion but also to sedimentation and agglomeration or aggregation.[110] 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 as agglomerates, which are held together by van der Waals forces, hydrophobic interactions and/or hydrogen-bonds.[159] 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 NPs.[148,159] Thus, agglomeration occurs in media when attractive forces overpower the electrostatic or steric repulsion between NPs.[160] Since both diffusion and sedimentation are influenced by agglomeration, the latter is expected to have a major impact on the NP uptake and toxicity.[161] Therefore the concept of dose is more complex and dynamic for NPs and requires the modelling of the NP behaviour in dispersion.[110,126]

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. For example nanorods and fibres have been shown to agglomerate more easily than spheres and smaller NPs typically aggregate more than their larger counterparts at similar mass doses, which can be explained by the higher number density.[162,163] Several medium-related parameters like pH, salt composition, ion concentration and ionic strength were suggested by a number of groups to potentially influence NP agglomeration.[41,160,164] Additionally, the presence of proteins, in particular serum proteins, has been shown to be a very important factor.[160] Yet, its effect on NP agglomeration is not fully understood as several groups found conflicting data, showing either less or more agglomeration in serum containing media.[165,166]

Since the majority of the NPs are unstable in biological fluids, agglomeration is believed to be inevitable *in vivo*.[160] 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 both *in vivo* and *in vitro*. Certainly since aggregates show altered kinetics when compared to their single NP counterparts with the same size.[160] However, evaluating NP agglomeration in the applied cell medium or biological fluid is not an easy task. Dynamic light scattering (DLS) is a commonly used 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.[167] To overcome these obstacles, alternative methods have been put forward, such as the use of fluorescence correlation spectroscopy or fluorescent single particle tracking (fSPT), which allow the accurate and precise determination of the size distribution of fluorescent NPs in undiluted biological fluids.[167,168]

This section provides an overview of studies evaluating the influence of NP sedimentation on uptake and toxicity as well as innovative setups to avoid the effect of sedimentation *in vitro*.

4.1 Effect of nanoparticle agglomeration and sedimentation on uptake

Several studies showed that the impact of agglomeration is not very straightforward, as aggregates show either enhanced or impeded uptake when compared to uptake of single NPs.[162,163,165] The higher uptake levels for aggregates can be explained by the fact that they reach the cells more rapidly by sedimentation, while the transport rate of single NPs is limited by diffusion.[163] However, when the agglomerate size is similar to, or larger than the cell itself, uptake is impeded by physical restrictions to the uptake processes.[162,165] These observations support the assumption that larger aggregates do not enter the cell *via* the same mechanism as single NPs or small agglomerates, as most common endocytosis routes like clathrin- or caveolin-mediated endocytosis are limited to the uptake of materials with dimensions of maximally 80 nm.[165] Furthermore, NP uptake mechanisms were found to be not only size- but also cell type-dependent, even for non-phagocytotic cells.[160] Additionally, each NP will agglomerate in a specific way, which implies that the NP type must also be taken into account when evaluating the influence of agglomeration on NP uptake.[141]

From the available (conflicting) data it cannot be concluded whether single NP or small agglomerates are taken up to a higher or lesser extent, but we do hypothesize that the 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 therefore 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. 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

Since NP uptake is influenced by agglomeration and sedimentation, it was hypothesized that NP cytotoxicity might also be affected. Similarly to NP uptake, the influence of agglomeration on nanotoxicity levels is not straightforward. On the one hand clear correlations have been found between NP concentration, aggregation, precipitation and cell injury for different types of QD and carbon nanotubes (CNTs).[36,169] In contrast, other studies show reduced cytotoxicity for agglomerated NPs, due to the fact that smaller entities are in general taken up more avidly by non-specialized cells and more easily reach intracellular structures such as the cell nucleus or mitochondria, which are less accessible to larger particles or aggregates.[162,170,171] 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_3 NP and agglomerate toxicity in suspension 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.[172]

These data clearly show the influence of NP sedimentation on *in vitro* nanotoxicity studies 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 NP rather than reaching unrealistically high cell exposure levels due to NP sedimentation.

4.3 New model systems to minimize sedimentation effects

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.

4.3.1 Inverted models. Cho *et al.* developed an inverted cell model in which they evaluated the influence of sedimentation on uptake of Au nanospheres, -cages and -rods in human breast cancer cells (SK-BR-3). This is an elegant and straightforward cell model in which sedimentation itself is not avoided but will not result in increased cellular exposure levels for cells that are cultured in the inverted configuration (cells grown on an insert at the top of the medium, facing downwards). The authors observed a much more avid uptake of NPs in classical cell cultures in comparison to cells cultured in the inverted configuration (Fig. 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. The differential uptake in upright or inverted configuration depended on the physical properties of the NPs and was most distinct for NPs with a greatest propensity to sediment, underscoring the necessity to avoid the influence of sedimentation.[34]

4.3.2 Flow and microfluidic models. More complicated models are flow or microfluidic systems where gravitational setting is impeded or even completely avoided. The applied flow also assists in acquiring a more homogeneous NP distribution and is believed to alter the cell–NP contact time.[36,121] Additionally, applying a flow implies the continuous renewal of medium,

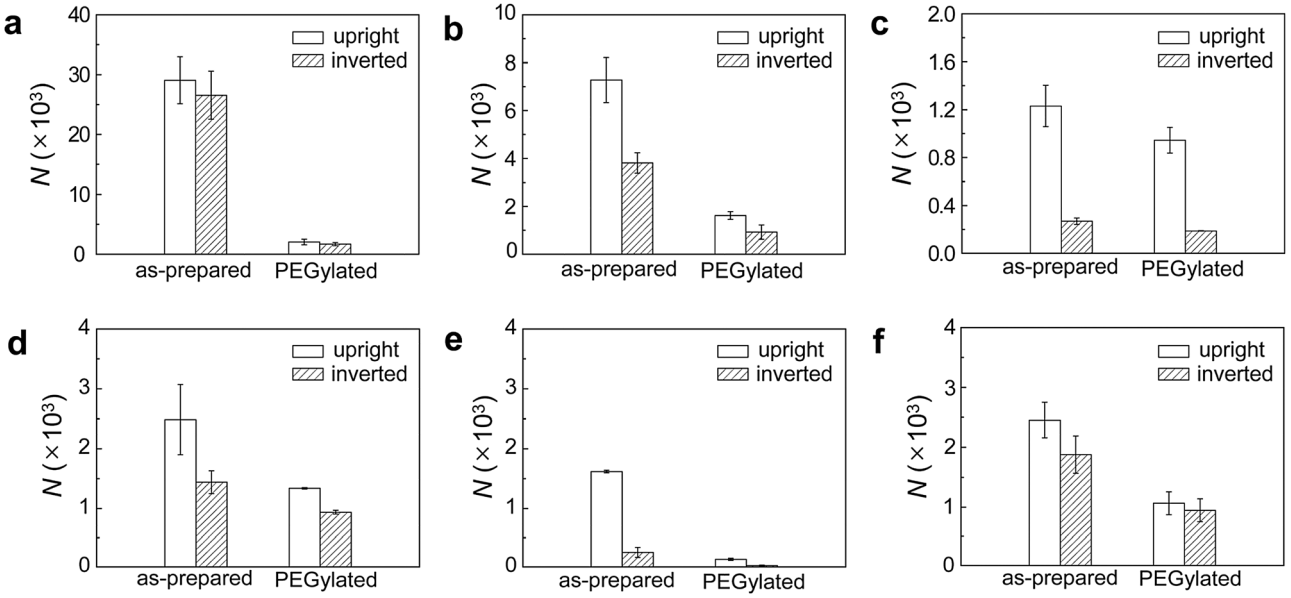

Fig. 5 The effect of cell culture configurations (normal horizontal (= upright) *versus* hanging down (= inverted)) on the uptake of various types of NPs. Differential uptake of (a) 15 nm nanospheres (120 pM); (b) 54 nm nanospheres (20 pM); (c) 100 nm nanospheres (2.8 pM); (d) 62 nm nanocages (20 pM); (e) 118 nm nanocages (2.6 pM); and (f) nanorods (20 pM) in the upright and inverted configuration. This figure is reproduced with permission from Cho *et al.*[34] © Nature Publishing Group 2011.

which ensures a sustained supply of nutrients and a constant pH.[35] We believe that the main advantage is that cells show a more *in vivo*-like behaviour as a laminar flow can activate endothelial cells and suppress proliferation, apoptosis and ROS induction.[173] Cellular morphology is also altered as the shear stress (SS) induces elongation of endothelial cells in direction of the flow and formation of actin stress fibres, clustering around the nucleus, to protect the cells from hemodynamic damage *in vivo* as well as *in vitro*.[173–175] Samuel *et al.* furthermore observed membrane blebbing under flow conditions, whereas cells under static conditions showed a smooth and flattened morphology.[175] Taking these aspects into account application of a flow provides a more dynamic and *in vivo*-like model.

Several groups compared NP uptake under flow and static conditions and typically found higher uptake levels under static conditions, due to the contribution of NP agglomeration and sedimentation.[174,176] However, the plethora of effects of a fluid flow on cell morphology and function can have a major effect and might, in contrast to the general findings, result in enhanced NP uptake. For instance, Samuel *et al.* observed uptake of untargeted NP in cells from a HUVEC cell line (CRL-1730) under flow conditions but not in the classical static 2D cultures.[175] They also did not find any difference in NP uptake when CRL-1730 cells were activated or not, while other studies clearly show higher uptake for targeted NPs in activated cells.[174–176]

Some groups subsequently explored the influence of the level of applied SS on NP uptake. Most studies found the highest levels of adherence and uptake under the lowest SS, as NPs are more likely to sediment on top of the cells under these conditions, which prolongs the cell–NP contact time.[177,178] The same effect was observed for targeted NP *in vivo* in mice, where this effect was attributed to impaired endocytosis at higher SS due to the increased stiffness of the cytoskeleton.[174]

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 following a 12 h exposure time: cells in static condition showed approximately 30% cell viability, whereas the percentage of living cells under SS remained 75% and significantly fewer cells were deformed or detached.[35] 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 2 h exposure to unmodified SiO_2 NPs. They accounted this difference to the possible activation of the HUVECs under higher SS.[121] But we believe that the shorter incubation time must also be taken into account.

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 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 known to influence NP uptake and toxicity. It is observed that non-targeted NPs are taken up more avidly under flow conditions, while functionalised NPs show higher uptake under static conditions. Nanotoxicity results are on the contrary conflicting, so no firm conclusions can be drawn on this subject yet.[35,121] But as toxicity levels measured in these novel model systems significantly differ from those obtained from classical static 2D monocultures, which are believed to be

less *in vivo*-like, we believe that flow models should be optimised and that 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 NPs.

5 The effect of cell communication

Another important shortcoming of the monoculture model is the lack of intercellular communication. This crosstalk between different cell types is known to be vital in sustaining homeostasis and in complicated processes like the processing of xenobiotics, inflammation and immune responses, which can consequently hardly be accurately modelled in monocultures.[179,180]

Several groups have focussed on establishing co-cultures in order to overcome this shortcoming and 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 separated by culture inserts. Cells co-cultured on culture inserts have a more differentiated phenotype, develop tight and adherent junctions and are polarised, implying that the cells have an apical and basolateral membrane with a distinct composition.[181,182] Thus, these models mimic the *in vivo* environment more closely and are therefore assumed to have a higher predictive power. This section gives an overview of studies evaluating NP uptake and toxicity in these novel model systems.

5.1 Effect of intercellular communication on NP uptake

Since it is well known that the majority of NPs are rapidly taken up by the reticulo-endothelial system after intravenous administration, it was investigated whether the same trend could be observed in co-cultures of epithelial and phagocytotic cells. In general the highest and lowest uptake levels are respectively found in macrophages and epithelial cells.[183] Rothen-Rutishauser *et al.*, for example, evaluated polystyrene, Au and TiO_2 NP uptake in a co-culture consisting of A549 cells, human monocyte derived macrophages (MDM) and human monocyte derived dendritic cells (MDDC) and observed preferential uptake of all NPs in MDM and the lowest uptake levels in A549 cells (Fig. 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.[184] Next to the effect of cellular phagocytosis, it has also been observed that co-culturing cells can result in further differentiated and polarised cells that show a more barrier-forming phenotype, which impedes NP uptake.[181]

It can be concluded from these data that NP uptake is significantly altered in co-cultures, with the two main factors influencing differential NP uptake being (i) the level of cellular differentiation and (ii) the combination of cell types and associates with this the preferential uptake mechanism, especially when phagocytic cells are included.

5.2 Effect of intercellular communication on NP toxicity

The uneven distribution of NPs in different cell types in a co-culture implies that the NP dose will not be equal in all cell types, but is for instance likely to be higher in the macrophages and lower for other cell types in comparison to the intracellular dose in monocultures. It was therefore hypothesized that nanotoxicity found in co-cultures would significantly vary from that in monocultures.

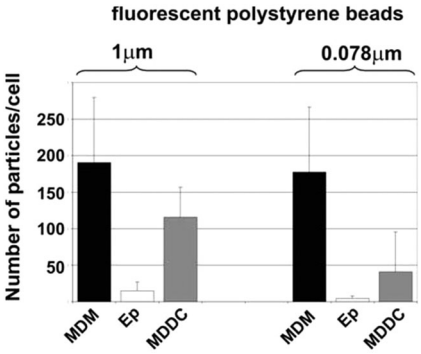

Fig. 6 Preferential uptake of fluorescent polystyrene beads in human monocyte derived macrophages (MDM) in co-culture with A549 cells (EP) and human monocyte derived dendritic cells (MDDC). This figure is reproduced with permission from Rothen-Rutishauser *et al.*[184] © Biomed Central 2007.

Since the lung is expected to be one of the major target organs of NP toxicity *via* the inhalation of airborne NPs, most studies are performed on co-cultures representing alveolar tissue. It has been found that particulate matter (PM_{10}) treatment causes this type of co-culture to secrete more cytokines than the sum of the cytokine secretion in the corresponding monocultures.[185,186] This increased cytokine production renders cells more susceptible to NP-induced damage, as was observed by Kim *et al.* who found the potential of 8 metal NPs to induce apoptosis in a RAW264.7/MLE12 (murine alveolar long-lived cell line) co-culture and both monocultures to be significantly higher in the co-culture due to increased TNFα release by the RAW264.7 cells.[187]

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 the NPs. This model allows the evaluation of the potential of NPs to activate endothelial cells through intercellular communication, which can subsequently induce systemic effects. Using this model, several studies revealed that co-cultures are more resilient to nanotoxicity in terms of acute toxicity but show an increased inflammatory response.[181,182,188] Ramos-Godinez *et al.* did, on the contrary, not find any changes in cytokine release for a A549/HUVEC co-culture after TiO_2 NP exposure. However, HUVECs from the co-culture did 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, implying that endothelial cells were activated *via* intercellular communication in co-cultures.[189] In none of these studies NPs were found to cross the culture insert, possibly due to the barrier formed by the epithelial cells or agglomeration of the NPs. This supports the hypothesis that endothelial cells were activated through intercellular communication after exposure of epithelial cells to NPs.

Additionally, it has been shown that the activation status of macrophages has an influence on nanotoxicity since non-activated macrophages act as a reservoir, thus performing a protective function while activated macrophages elicited secondary toxicity in the accompanying cell type *via* cytokine excretion.[190,191] According to van Berlo *et al.* the protective or aggravating effect on NP toxicity furthermore depends on the combination of cell types as he found macrophages to protect A549 cells against oxidative DNA damage whereas the neutrophils aggravated the effect.[192]

Additional cell types can be added 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. They found that all cultures had a distinct cytokine excretion profile with the cytokine production being either amplified or mitigated in co-culture. This was explained by the differential expression of cell surface receptors depending on the cell type or changes in crosstalk between the cells by addition of an extra cell type.[193] Besides the cytokine excretion, Muller *et al.* also found the total antioxidant capacity to be modulated in a A549, MDC and MDDC co-culture.[194] This underscores the importance of evaluating nanotoxicity in co-cultures since both the inflammatory response and oxidative stress levels are known to be influenced by NP treatment.

To summarize we can conclude 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, 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 *in vitro* nanotoxicity assessment since these models are more *in vivo*-like and therefore more relevant and likely to have a higher predictive power and allow the study of effects that could never be picked up in monoculture experiments.

6 The effect of a 3D environment

As discussed in Section 2.2.3 is the loss of the specific 3D tissue architecture and cell polarisation a final major shortcoming that will be discussed in this review. An additional advantage to 3D cultures, besides the more *in vivo*-like phenotype and ECM production, is that cell–cell and cell–matrix communication are promoted due to the tighter packing of the cells in a 3D setup. In turn, the enhanced communication influences a number of important cellular functions such as migration, invasion, proliferation, apoptosis and differentiation.[195] 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 when evaluated in a 3D model.[124]

Another advantage of 3D models is the fact that cultivation times of most cells can be prolonged in comparison to 2D cultures, which enables long(er)-term *in vitro* experiments.[196] Therefore, 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.

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.[197] However, hydrogel-based scaffolds are likely to be not very useful for NP studies as thick scaffolds may limit the diffusion of NPs towards the cells, which will drastically alter the outcome of any uptake or toxicity study. Initially many research groups applied 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 as will be described in the section below.

6.1 Effect of a 3D environment on NP uptake

Since cells cultured in spheroids are known to produce a more dense and complex ECM, several groups compared NP uptake in a 2D and 3D setting. In comparison to classical 2D monocultures where NPs are evenly distributed, they show an uneven distribution and limited penetration in spheres.[195,198] Consequently, NP uptake is generally lower and found to be restricted to cells in the peripheral layers of the spheres due to the protective effect of the ECM.[195] An interesting study by Huang *et al.* compared 2; 6 and 15 nm diameter Au NP uptake in a MCF-7 monolayer, spheroid model and mouse breast tumours *in vivo*. The spheroids and tumours showed the same trends in tissue penetration as 2 and 6 nm NPs could penetrate the deeper regions of the cell mass, but 15 nm NPs were only found in the periphery, while all NPs were evenly distributed in the monolayer.[199]

Thus, it is generally seen that NP distribution is less homogeneous in 3D cultures. The ECM furthermore hampers NP penetration into a 3D matrix, which limits NP uptake into the cells in the peripheral layers of the 3D culture, especially for larger NPs.

6.2 Effect of a 3D environment on NP toxicity

Several groups evaluated NP toxicity simultaneously in 2D and 3D cultures to assess whether cellular responses to NP exposure are also drastically altered in a 3D environment. In general, the toxicity induced by NP enclosed anti-cancer agents was found to

be significantly lower (5- to 20-fold) in spheroid models than in the classical monolayer.[195,198] In one of the first studies using 3D models for nanotoxicity purposes Lee *et al.* evaluated 2.9 nm diameter CdTe QD and 3.5 nm diameter Au NP toxicity in HepG2 2D and spheroid cultures and found a substantially lower toxicity in the latter. 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 (Fig. 7). Longer exposure times induced more severe damage to the cells in the periphery of the sphere while the interior remained unimpaired. When the same incubation conditions were applied to 2D cultures, they observed more overall cell death (Fig. 8). These effects were attributed to the protective effect of the barrier consisting of the ECM 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.[200] Various other groups confirmed these results, as for instance CdSe/ZnS QD, IONP and SiO_2 NP toxicity in HeLa microspheres was radial and lower in the 3D setup due to the protective effect of the surrounding cells.[125,201] On the contrary, Yu *et al.* found 5 and 30 nm diameter IONPs to cause more severe toxicity, starting at lower doses to porcine aortic endothelial cells in a 3D alginate matrix than to 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 72 h exposure time, cells in 2D cultures were likely to be exposed to lower doses of IONPs as the NPs were diluted upon cell division. This protective effect was likely to be impeded in the 3D setup, possibly causing the cells to show higher stress levels and toxicity.[202]

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 way 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 sphere. These 3D models are likely to have a higher predictive power,

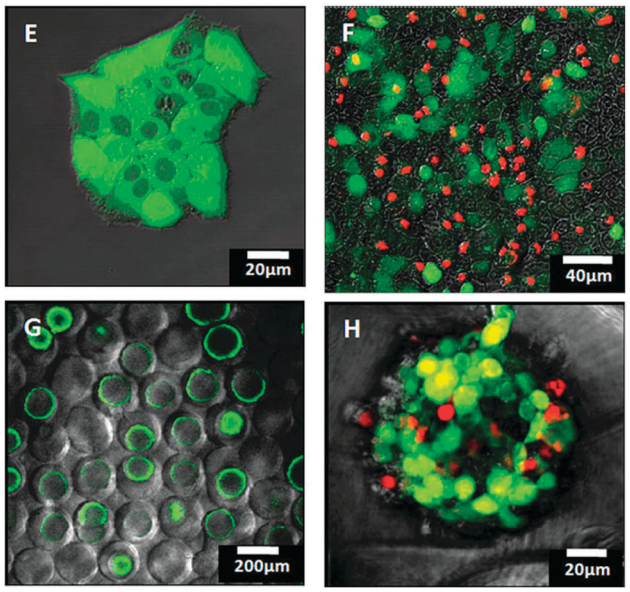

Fig. 7 The effect of NP toxicity in both a 2D and 3D cellular environment. Confocal images of live[69]/dead (red)-stained normal (E) 2D and (G) 3D spheroid cultures and after CdTe NP exposure in (F) 2D and (H) 3D spheroid cultures. This figure is reproduced with permission from Lee *et al.*[200] © Wiley-VCH 2009.

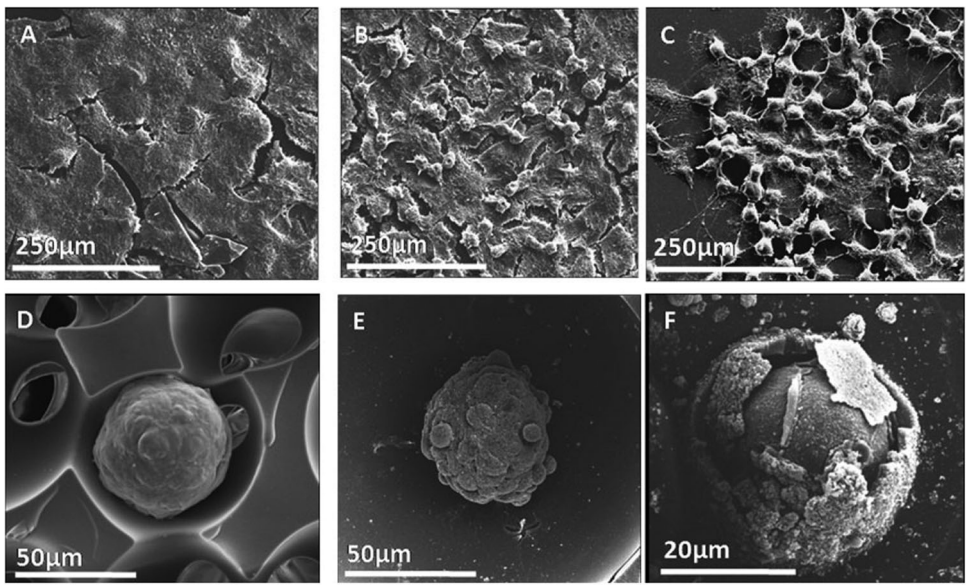

Fig. 8 Effect of NPs on cell morphology on 2D and 3D cell cultures. SEM images of 2D and 3D spheroid cultures before and after CdTe NP exposure. Typical morphology of (A) 2D and (D) 3D spheroid cultures after 5 days without CdTe NP exposure. Representative morphology of (B) 2D and (E) 3D spheroid cultures after 12 h of CdTe NP treatment. Morphological change of (C) 2D and (F) 3D spheroid cultures after 24 h of CdTe exposure. This figure is reproduced with permission from Lee *et al.*[200] © Wiley-VCH 2009.

as they are a closer representation of the *in vivo* environment. Therefore, we believe that the development of 3D models is a recent milestone in bridging the *in vitro–in vivo* gap and that these models are likely to greatly gain importance in the next few years. It is important for these models to reach full maturity that any results obtained are compared to both 2D cell monocultures and *in vivo* animal studies. Where data obtained in 3D models have been compared to *in vivo* data for small chemicals, for NPs, to our knowledge, this has not been done thus far, partially due to the low number of available data from animal studies.

7 Conclusion and perspectives

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 prior to 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 thorough evaluation of nanomaterial–cell or –tissue interactions is greatly impeded by the numerous types of NP (each with their specific features) and the heavy impact of even the most miniature changes to a single physicochemical parameter on the NPs interaction with biological entities,

The most used *in vitro* model, namely the classical 2D monoculture, is a very reductionist approach where most of the complexity of the *in vivo* situation is lost. Therefore, results from *in vitro* studies often did not relate very well to findings obtained in *in vivo* studies. Several groups have therefore made substantial efforts in trying to optimize the current *in vitro* models to mimic the *in vivo* conditions more closely. Inverted cell cultures, flow models, co-cultures or 3D cell cultures all have their advantages for NP uptake and toxicity studies when compared to the classical 2D monolayers as described in the various sections above.

Since the field of nanotechnology keeps blooming and the safety of nanomaterials remains questionable as we are all being exposed more and more, it is 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 on NP safety should be possible as well as a better selection of materials that are more interesting to further *in vivo* evaluation. 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 is the possible combination of several of these models. Using co-culture models or flow models in a HCS setting would enable these methods to be used as rapid screening tools. Additionally, the use of 3D models or co-cultures in combination with the dynamic flow would provide many opportunities to further bridge the *in vitro–in vivo* gap and rapidly study the targeting efficacy of, for instance, anti-cancer agent-containing NPs 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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