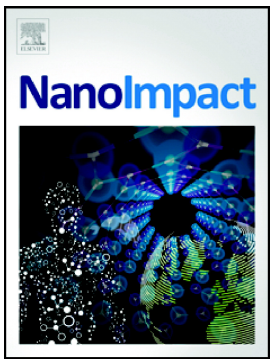


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# REVIEW ARTICLE

# Refining *in vitro* models for nanomaterial exposure to cells and tissues

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## Abstract

With the increasing use of nanomaterials (NMs) in a variety of commercial and medical applications, there is a parallel increase in concern related to unintentional exposure. This leads to a pressing need for appropriate hazard and risk assessment, and subsequent regulation of these new and emerging nanosubstances. Typically, *in vitro* models are the first point for assessment, and these are often then used to begin to predict and translate the potential effects *in* vivo. The area of nanotoxicology is therefore critically important, and requires that experimental protocols are clear, defined and standardized within adequate risk assessment frameworks to allow hazard identification and extrapolation to more realistic *in*

*vivo* situations. Often, however, results seen *in vitro* do not adequately represent the situation *in vivo.* There are many differences between *in vitro* and *in vivo* investigations; however one issue may arise from the way in which typical nanosafety studies are carried out. Nanotoxicity assessment requires a definition of dose and standardized modes of exposure in nanomaterial uptake and cytotoxicity studies to determine the hazard that is posed by naturally occurring and engineered (E-)NM. Current methods in nanotoxicity studies often report only the exposure dose, which can lead to large variations in the *intrinsic* or *delivered dose* due to inhomogeneous exposure and loss of treatment material depending on how NMs are "*presented*" to cells. Protocols for Nanomaterial (NM) dispersion and cellular assay design therefore require Standard Operation Procedures (SOPs). Many experimental conditions in NM studies directly affect the NM behaviour within the cell culture system, in particular handling of NM dispersions as well as the order, timing and exposure configurations of the incubation; these can have an immediate effect on the resulting physical distribution of NMs on the cell surfaces. Here we review handling, physical conditions and particle distribution in cell models for NM exposure to cells and tissues. Efforts to improve *in vitro* models such that they more closely mimic the *in vivo* conditions, such as exposure mechanism and dose, and potential for transport of nanomaterials across cells are being developed rapidly, driven partially by the strong push within the EU to reduce animal testing. Examples include the use of multiple *in vitro* assays to calculate hazard, air-liquid interface (ALI) exposure, microfluidic approaches and 3D co-culture models.

**Keywords**

Nanosafety, nanomaterials, reproducibility, dosimetry, presentation mode, 3 dimensional cell culture, organoids, receptor mediated endocytosis, diffusion, sedimentation

**List of abbreviations:**

3D- 3-dimensional; ALI – air-liquid interface; BBB – blood brain barrier; BSA – bovine serum albumin; DCS - Differential Centrifugal Sedimentation; DLS - dynamic light scattering; DSE – delivered sonication energy; DLVO – Derjaguin, Landau, Ververy and Overbeek theory; (E-)NM – engineered nanomaterial; FAE - follicle-associated epithelium; FBS - foetal bovine serum; GI - gastrointestinal; IATA - Integrated Approaches to Testing and Assessment; ISDD - *In vitro* Sedimentation, Diffusion and Dosimetry model LM – Light Microscopy; MWCNT -

multi-walled carbon nanotubes; NM – nanomaterial; OECD – Organisation for Economic Cooperation and Development; PDE - partial differential equation; PCTS - precision cut tissue slices; QD - quantum dot; REACH – Registration, Evaluation and Authorisation of Chemicals; ROS – reactive oxygen species; SOP - standard operating procedure; spICP-MS – single particle Inductively coupled mass spectrometry; TEM – transmission electron microscopy; TfR - transferrin receptor.

## Introduction

Exposure to naturally formed nanomaterials (NMs) can occur through processes such as weathering, erosions, and volcanic eruptions. NMs can also be an integral part of natural biological processes such as the sensing of the earth's magnetic field by bacteria (Loehr *et al.*, 2016). However, the past decade has seen an unparalleled increase in the range and quantity of NMs produced commercially for use within a host of applications ranging from electronics, remediation, food additives and biomedical imaging and therapy. Therefore it is a critical priority that appropriate toxicological studies are performed to ascertain the potential short and long term risk posed by these emerging engineered (E-)NMs. Proper risk assessment is therefore necessary to ensure the safe use of these agents within the range of applications in order to avoid a repetition of catastrophic events in history that were seen with exposure to substances such as asbestos (Seaton *et al.*, 2010). An important factor to achieving this goal is the ability to at least partially translate the effects seen *in vitro* to the potential for harm *in vivo*, as the expense of *in vivo* testing makes extensive testing of all potential NMs impossible. Indeed, it has been estimated that it will cost up to €600 million to register the 500 - 2,000 NMs that are expected to be placed on the EU market at volumes of at least 1 tonne/year under REACH and other legislations (e.g., for cosmetics), the vast majority of which is related to the need for 90-day inhalation and oral animal exposures (European Commission, 2013). Thus, there is an urgent need for *in vitro* methods that are predictive of the *in vivo* outcomes observed, in order to reduce our reliance on animal testing. This could comprise a battery of *in vitro* assays that should be performed to collectively inform on the (E-)NM potential for harm, or specific testing strategies incorporating 3D models or specialized exposure systems. It is important to note that these in vitro approaches that are discussed herein provide a means for addressing the hazard component of a risk assessment. Once suitable Standard Operating Procedures (SOPs) are developed for the hazard assess-

ment of (E-)NMs, these can be incorporated into a risk assessment framework to appropriately determine the potential for harm based on environmentally relevant expected exposure levels.

*In vitro* toxicity testing is therefore a fundamental part of any hazard identification strategy. An essential component of making these necessary toxicological studies reproducible and predictive of the *in vivo* situation is the availability of standardized assays to allow reliable determination of the toxic potential. A number of factors can determine the toxicity associated with a given NM; the actual uptake behaviour of NMs is one key parameter, and as such is a much studied area (Aillon *et al.*, 2009; Alkilany and Murphy, 2010; Misra *et al.*, 2012; Panariti, Miserocchi and Rivolta, 2012; Huk *et al.*, 2014; Kettler *et al.*, 2014; Shin, Song and Um, 2015). Therefore, the properties that determine toxicity are often determinants in the amount and mechanism of cellular entry of the specific NMs and their eventual effects within the cell population; this is discussed more in the section 'cellular uptake of NMs'. Many current *in vitro* toxicity assays applied to NMs are therefore based upon NM exposure studies followed by determination of toxicity by end-points such as induction of reactive oxygen species (ROS), lysosomal integrity or membrane permabilisation (Farcal *et al.*, 2015).

There are a number of variables that can lead to differences or inaccuracies during these *in vitro* procedures that arise both from the experimental *in vitro* system used and the NM suspensions themselves, which will be introduced in the subsequent sections. For example, standard toxicity assays were generally designed for use with chemical compounds or molecules, therefore they may well not be appropriate for assessment of NM toxicity – some NM have even been seen to bind to standard dyes used in these types of assays (Ong *et al.*, 2014). Furthermore, NMs present in suspension and/or bound at the cell surface can also interfere directly with the read out by altering the light absorbance or scatter due to their optical properties (Bahadar *et al.*, 2016). Various interferences have been reported between NMs and standard toxicity assays which can confound toxicity assessment results (Kroll *et al.*, 2009). Minimising these types of interferences is therefore critical for successful *in vitro* assays, and often relies on the determination of appropriate control experiments to perform alongside (Rosslein *et al.*, 2015). Even when performed alongside appropriate controls, a single assay can often not give enough information regarding the toxic potential of some

NMs. There has therefore been a significant push to find assays or combinations of assays that can better reflect the *in vivo* hazard posed by NMs with some success (Wiemann *et al.*, 2016). The macrophage assay, whereby alveolar macrophages are treated with numerous different NMs, involves the subsequent measurement of multiple endpoints that can then be used to determine the active or passive nature of NMs; the assay prediction models proved easy to use, and highly efficient for predicting *in vivo* hazard potential of NMs (Wiemann *et al.*, 2016). Similar multi-endpoint principles were applied in the high throughput screening of NMs using microscopical examination as part of the FP7 NanoMILE project (Hansjosten *et al.*, 2017). In these examinations multiple endpoints were assessed and then NMs were scored based on the result severity, giving a multi-parametric approach to advise on the various hazards associated with cellular exposure to a variety of NMs (Hansjosten *et al.*, 2017). Indeed, the integration of data types from several testing strategies (old or new) toward NM hazard characterization is the basis of the OECD Integrated Approaches to Testing and Assessment (IATA), although regulatory acceptance of newly emerging approaches lags considerably behind their development (Sewell et al., 2017).

As the field of nanotoxicology progresses there is an increasing demand for a higher level of reliability and quantification of the assays currently performed. Recently, Rosslein *et al.* described the use of cause and effect analysis of assay systems to determine sources of variability within tested methods. This was aimed at establishing the necessary controls in order to increase the validity of *in vitro* results (Rosslein *et al.*, 2015). This cause and effect method allows for the use of controls that can account for some of these unwanted interactions and effects, such as NM interference and NM binding to assay components (Ong *et al.*, 2014; Guadagnini *et al.*, 2015; Rosslein *et al.*, 2015). Methods such as these are very important for developing SOPs, and can be used to greatly enhance the reliability of the data, accounting for the large number of variables present including cell seeding and instrument interference. Where experiments do not reflect similar results, increased recording of crucial metadata surrounding experimental conditions from plating to end-point determination will undoubtedly play an important role. This metadata might also indicate critical factors that determine reproducibility, and as such should be specified in future versions of protocols. Recording of these steps, along with information on NM storage conditions and age/provenance, will likely minimize the variability between different studies performed at separate points in time and

locations and thus increase the reliability of the data available (Sarathy *et al.*, 2008; Kuchibhatla *et al.*, 2012; Izak-Nau *et al.*, 2015; Baer, Munusamy and Thrall, 2016). Sample ageing during storage also plays an important role, and indeed the same NMs tested 6 months apart for their effects on A549 (Adenocarcinomic human alveolar basal epithelial ) cells resulted in significantly different toxicity, even when the NM stock solutions were stored under "optimal" conditions – i.e., at 4 °C in darkness (Izak-Nau *et al.*, 2015). Thus, NM 'aging' effects can be a significant contributor to the contradictory toxicity results observed in the literature for identical NMs, and NM ageing should be assessed in parallel with toxicity assessment as best practice.

Despite the advances in terms of assay reliability and availability, often the performed *in vitro* studies give rise to conflicting information and poorly reflect the situation *in vivo.* It is likely that inappropriate NM dispersion, poor characterisation of the NMs dispersions over the exposure duration, incomplete reporting and use of incorrect exposure metrics in *in vitro* studies play a largely ignored role in the poor *in vitro-in vivo* correlations to date (Hinderliter *et al.*, 2010; Dawson *et al.*, 2013). One fundamental principle in toxicology is the "dose-response" whereby the effect of a molecule is directly proportional to the dose at the specific target site. However, the dose-response is not always linear for NMs – higher doses can lead to agglomeration, via weak interacting forces between NMs, and thus less available dose and lower toxicity (Baalousha, 2009; Hussain *et al.*, 2009; Mudunkotuwa and Grassian, 2011; Bell, Ives and Jonas, 2014). It is important here to distinguish between reversible NM agglomeration, and the stronger irreversible formation of aggregates, as the two terms are often, incorrectly, used interchangeably (Sokolov *et al.*, 2015). Another important aspect is the way in which the dose is quantified and reported. There is no official or standard definition of NM dose as of yet, and often it is recommended that multiple doses are reported including mass, number and surface area concentration (Drasler *et al.*, 2017). Often the dose is given as the mass of NM per area cell culture in μg/cm$^2$. Sometimes the dose is only indirectly defined by specification of the NM concentration in μg/mL administered to the cell culture. The implications of dosimetry and particokinetics on the interpretation of response was first highlighted by Teeguarden et al, and has since been the subject of comprehensive reviews (Teeguarden *et al.*, 2007a; Park *et al.*, 2009; Drasler *et al.*, 2017). The measure of the dose at a specific site will depend largely on the experimental conditions and therefore these

effects must be accounted for to ensure accuracy of the reported results. The cellular NM dose is often assumed to be estimated by, or proportional to, the concentration of NM in the treatment media in *in vitro* studies – a situation that is unlikely to be the case for numerous reasons, including NM agglomeration, loss to vessel walls (Figure 1), and the fact that the uptake of NMs is generally receptor mediated such that only a proportion of the available NMs can be internalised at any moment in time (Figure 2). Work on single particle identification and tracking of NM uptake renders the most obvious definition of NM dose as the number of NM per cell (either internalized or tightly bound) (Drasler *et al.*, 2017). This can be referred to as the "local dose" or "cellular dose", in contrast to the quantity of the administered material which can be thought of as the "Administered dose" (Richitor *et al.*, 2016).

Differences between the administered and local cellular dose can arise through a variety of means, including, but not limited to, alterations in size distributions due to agglomeration, changes in total NM number due to loss of material (e.g. for NMs that undergo dissolution), transport of the NM through the medium and inhomogeneous distribution. Here, the administered dose is discussed in terms of its relation to the local (cellular) dose by evaluating the evolution, transport and distribution of NMs during preparation and cellular exposure. Within this context, this review evaluates methods for performing NM uptake experiments and reports on the distribution of NMs over a cell population from the different approaches. There are a variety of modes available for NM presentation to cells (see Figure 3) and with correct reporting and performing of the experiments these can provide reproducible and accurate comparisons between NM studies in adherent cell types *in-vitro*. The goal of the evaluation made here is therefore to formulate a recommendation for the standardized delivery of NMs to cultured cells and the minimum amount of information that should be reported. Indeed, within the EU-funded QualityNano research infrastructure, a central goal was the development and ring-testing of a number of SOPs for NMs physico-chemical characterisation and *in vitro* toxicological evaluation, including NM size characterisation by Dynamic Light Scattering (DLS) and Differential Centrifugal Sedimentation (DCS), quantification of cellular uptake of NMs by flow cytometry and cytotoxicity determined by the MTS assay, which included detailed descriptions of cell culture processes and the dispersion of the NMs in the appropriate cell culture medium. These protocols, and the ring-tests or interlaboratory comparisons undertaken on them, are described in detail in articles included in this special issue

(Langevin et al. 2017; Salvati et al. 2017).

## NM dispersions and agglomeration

Prior to *in vitro* toxicity studies, NMs must first be dispersed in aqueous media. We have shown that, without detailed instructions for sample preparation and measurement, even for simple measurement of NM dispersions in water, high variability in size and size distribution can be generated (Langevin *et al.*, 2017). For this reason there has been a large focus on obtaining SOPs for repeatable dispersion methods using inter and intra-lab comparisons, developed as part of EU Framework Programme (FP6 and FP7) funded projects (Farcal *et al.*, 2015). Obtaining a reproducible and homogenous dispersion in the complex biological fluids used for *in vitro* testing poses further challenges that have been addressed as part of the NanoGenoTox project, where an SOP was developed for the dispersion of hydrophobic NM powders such as $TiO_2$ and ZnO by using an ethanol pre-wetting stage, followed by dispersion in 0.05% BSA water and subsequent sonication and dispersion in cell culture medium (Alstrup Jensen *et al.*, 2011; Farcal *et al.*, 2015). The method of NM dispersion and the physiochemical properties of the dispersion can greatly affect the resulting outcomes in biological testing. Successful NM dispersion can be thought of as uniform, reproducible each time the process is applied (i.e. the same material dispersed the same way gives the same size and polydispersity index), stable over the duration of the test or characterised at the beginning and end of the exposure, and preferably consisting chiefly of primary particles with minimum size and minimum agglomeration. Unstable suspensions may have larger size distributions (more polydisperse), and altered agglomeration state (weak bonds between particles) and/or aggregation (harder to break bonds between particles) and tend to be less reproducible from one dispersion to another. This can lead to changes in the properties of the dispersion, and can therefore modulate processes such as cellular uptake. Larger particles are generally internalised less than smaller particles by receptor mediated processes and/or are internalised via different pathways (e.g. macropinocytosis rather than clathrin or caveolin mediated endocytosis) (Doherty and McMahon, 2009; Sahay, Alakhova and Kabanov, 2010; Zaki and Tirelli, 2010; Kou *et al.*, 2013; Kafshgari, Harding and Voelcker, 2015). Thus, the size and agglomeration state are important parameters when determining the potential toxicity of the material. NM dispersions will always show a certain level of polydispersity (typically between 0.1 and 0.4 is considered moderately polydisperse, with values >0.4 being very polydisperse,

although it should be noted that different synthesis routes can produce different polydispersities for the same NM), which can vary with material, solvent and synthesis method used, as well as with the stabilisation mechanism, e.g. sterically stabilised with polymer coatings versus electrostatically stabilised with small charged molecules such as citrate. However some NM preparations will show increased polydispersity due to agglomeration and inter-particle interactions, especially in media with high ionic strengths which can overcome electrostatic stabilisation. Much effort has therefore been focussed on studying the colloidal forces governing NM deposition and aggregation (Weitz and Lin, 1986; Petosa *et al.*, 2010). There are various methods that can be employed to reduce agglomeration effects during dispersion protocols, including ultrasound, milling and magnetic fields (Bihari *et al.*, 2008; Stuyven *et al.*, 2009; Vippola *et al.*, 2009). Derjaguin, Landau, Ververy and Overbeek (DLVO) theory of colloidal stability describes the agglomeration of aqueous dispersions and the active forces involved such as van der Waals forces and repulsion. Together with the interactions considered in the DLVO theory, forces such as steric interactions, magnetic forces (for iron-based NMs), and hydration forces influence the stability of NMs in aqueous media (Petosa *et al.*, 2010). Aggregation dynamics have also been found to be dependent on the amount and type of NMs (Weitz and Lin, 1986).

Several quantitative and qualitative studies have been performed to investigate NM aggregation, in addition to studies aimed at determining the most effective dispersion methods (Sano and Okamura, 2001; Anderson and Barron, 2005; K. L. Chen, 2006; Bihari *et al.*, 2008; Saleh, Pfefferle and Elimelech, 2008; Vippola *et al.*, 2009; Domingos, Tufenkji and Wilkinson, 2009; Fang *et al.*, 2009; Jiang, Oberdörster and Biswas, 2009; Bouwmeester *et al.*, 2011; Lamberty *et al.*, 2011; Ramirez-Garcia *et al.*, 2011; Mejia *et al.*, 2012). Sonication followed by the addition of dispersion stabilizers, such as Bovine serum albumin (BSA), serum or phospholipids has previously been determined as successful, and standardized methodologies describing this are therefore available (Bihari *et al.*, 2008; Alstrup Jensen *et al.*, 2011). An important factor that has been highlighted is the calibration of the Delivered Sonication Energy (DSE), both in terms of the energy delivered, and the material-specific critical DSE (crDSE) energy required to achieve stable suspensions with minimal aggregate size (DeLoid *et al.*, 2017).

Agglomeration leads to a large NM size distribution (polydispersity) and results in changes to the suspension, such as modification of the number of NMs present and the surface area that is available (DeLoid *et al.*, 2017). Different sized particles will also diffuse through liquids at different rates. Larger NMs, agglomerates and aggregates arrive at the cell surface at a faster rate in traditional submerged culture than smaller NMs due to the role of sedimentation (which is proportional to the square of their diameter), as discussed in the next section and shown in Figure 4 (Hanarp *et al.*, 2001). Agglomeration effects can be more pronounced when NMs are dispersed in protein rich cell medium at physiological pH and salt concentrations, due to protein corona formation which, depending on the NMs and the surrounding conditions, can lead to stabilisation or destabilisation of NMs dispersions (Monopoli *et al.*, 2012; Lynch *et al.*, 2013; Nasser and Lynch, 2016). Crist *et al*, highlighted the importance of performing characterizations in the appropriate media due to these changes that occur to the NMs properties (Crist *et al.*, 2013). They also highlighted the problems associated with using a single method of size characterization. For example, when examining Transmission Electron Microscopy (TEM) measurements of size, pre and post dispersion in cell media, the measured size remains the same (since TEM only measures the core electron dense particles not any surface adsorbed weakly scattering biomolecules), whereas when measured by DLS the size is nearly doubled as a result of biomolecule adsorption and hydration, indicating the necessity of multiple size measurements prior to *in vitro* toxicity studies (Crist *et al.*, 2013). Our recommendation is to combine a method to measure the core particle size (e.g. TEM), and a method to indicate the hydrodynamic size (in the exposure medium) such as DLS or DCS which provides an effective size in protein-containing medium (Domingos *et al.*, 2009; Dawson *et al.*, 2013). NM size distribution is most often measured immediately after NM exposure to cell medium. However, agglomeration is a time dependent process and the size distribution may change over the cell incubation period, and should therefore be monitored throughout the exposure duration (Shapero *et al.*, 2011; Dawson *et al.*, 2013). The NM size distribution at the cell surface will also differ from the size distribution in solution due to concentration effects. Overall, it is now widely recognised that NM uptake and impact studies require a detailed characterisation of the stability and properties of the NM dispersion. Therefore efforts are being made to define the minimum set of information and conditions which should be provided prior to cellular exposure experiments, which we have attempted

to summarise at the end of this article (Bouwmeester *et al.*, 2011; Roebben *et al.*, 2011; Crist *et al.*, 2013; Stefaniak *et al.*, 2013; Sharma *et al.*, 2014; DeLoid *et al.*, 2017).

## The choice of metric - NM dosimetry

There has been significant debate in the scientific literature as to the most relevant dose metric for NMs, with consensus that mass based doses are not relevant for NMs, while surface area and particle number seem more appropriate and more predictive of toxicological outcomes (Oberdörster, Oberdörster and Oberdörster, 2007; Wittmaack, 2007, 2011; Lison *et al.*, 2008; Rushton *et al.*, 2010; Simkó, Nosske and Kreyling, 2014; Braakhuis *et al.*, 2015; Schmid and Stoeger, 2016). Indeed, the same *in vitro* toxicity data for silver (Ag) NMs plotted by mass, surface area and particle number resulted in quite different outcomes. Results expressed in mass unit [$\mu g/cm^2$] suggested that the toxicity of Ag NMs was size-dependent, with 50 nm particles being most toxic to Chinese Hamster Lung (V79-4) cells. However, re-calculation of Ag NM concentrations from mass to surface area and number of NMs per $cm^2$ highlighted that 200 nm Ag NMs were the most toxic (Huk et al 2014). Wittmaack highlighted the necessity of reporting the cellular accumulated dose in a study of $SiO_2$ NMs, whereby the toxicity observed depended on the areal density of NM mass that is delivered to cells (Wittmaack, 2011). The importance of the dosimetry has led to the emergence of attempts to document and standardize the ways in which dosing is carried out and quantified, in the form of published protocols (DeLoid *et al.*, 2017). These protocols cover the generation of stable NM suspensions in culture medium, characterization of these colloids and modelling of the local dose delivered to cells over time (Hinderliter *et al.*, 2010; DeLoid *et al.*, 2017). These protocols include transport models that accommodate variable binding kinetics (of NMs) to the culture dishes, simulation of polydisperse suspensions and dissolution of NM during deposition to determine dose metrics for *in vitro* studies using MATLAB (DeLoid *et al.*, 2017).

Particle dosimetry differs significantly from that of conventional chemicals, partly due to the dynamic nature of particle suspensions which can be governed and modified by the NM physicochemical characteristics themselves and the properties of the surrounding media. This in turn leads to changes in the suspension itself and to the dose received by target cells in *in vitro* assays over the exposure time-course. Different NMs diffuse at different rates

dependent upon their characteristics such as size, density, shape, surface charge and surface coating/functionalization, discussed in more detail in the section 'Diffusion'. NMs can also agglomerate over time and acquire different surface constituents (charge neutralisation, corona evolution) which can also modify interactions with the surrounding fluid and cells. These evolving properties also lead to changes in sedimentation rates and therefore different amounts of NM reaching the target site, discussed later in the section 'Sedimentation'. The fluid itself leads to changes in NM interactions and motion, depending on parameters such as viscosity, protein content, ionic strength and pH, which are discussed more in the sections 'Diffusion' and 'Sedimentation'. Thus transport of NMs *towards the cell surface* can itself affects the NM distribution and cause formation of aggregates in *in-vitro* toxicity experiments as a function of time. The movement of NMs through a fluid is a combination of the sedimentation of the NMs and the diffusion of NMs over time, both of which are highly related to, and dependent upon, the agglomeration state and the dissolution potential in the media (see Figure 1). Mason and Weaver derived a mathematical solution to this, termed the laminar convection diffusion equation, in the form of a partial differential equation (PDE) as shown in equation 1 (Mason and Weaver, 1924). The components of this, sedimentation and diffusion, are described subsequently:

$$\frac{\partial n}{\partial t} = D\frac{\partial^2 n}{\partial x^2} - V\frac{\partial n}{\partial x} \qquad \text{(Equation 1)}$$

where *n* is particle concentration, *t* is time, *x* is distance and *D* and *V* are the Diffusion and Sedimentation velocities, respectively.

**Sedimentation**, or gravitational settling, is the tendency of particles to settle at the bottom of a container due to gravitation. This sedimentation rate, for single NMs, is a function of density of the medium, the density of the NMs in the medium, and diameter of the NM, and is described mathematically by Stokes' law (eqation. 2) which determines the gravitational settling velocity, *V* (Kajihara, 1971):

$$V = \frac{g(P_{Np} - P_m)d^2}{18\eta} \qquad \text{(Equation 2)}$$

where the acceleration due to gravity is $g$, $P_{Np}$and $P_m$are the mass density of the NMs and the medium respectively, $d$is the NM diameter and ŋis the cell culture medium dynamic velocity.

The process of sedimentation is shown schematically in Figure 4, along with the dependence of sedimentation on NM diameter and media and NM densities. Previously, variations of Stokes law, such as Sterling's modification, have been used to describe the sedimentation velocity of NM agglomerates, which will differ compared to that of single particles, and to determine the agglomerate effective density (Sterling *et al.*, 2005; Hinderliter *et al.*, 2010; Mukherjee *et al.*, 2014; Cui *et al.*, 2016). Agglomeration and aggregation are complex processes that are affected by the diffusion of individual and associated particles, in addition to the attractive and repulsive forces present between NMs. Sterling's modification for agglomerates incorporates properties of the agglomerates including their effective density, packing factor and fractal dimension, as agglomerates do not exist as homogenous units (Mukherjee *et al.*, 2014). These are important considerations when modelling NM transport, and therefore must be included in any calculations performed to determine the effective dose at the cell surface. However, Sterling's modification does not take into account the inter-particle forces, a factor that is incorporated into the previously mentioned DLVO theory (Petosa *et al.*, 2010). Sterling modification provides an estimate of the effective agglomerate density, rendering it potentially inaccurate (DeLoid *et al.*, 2014). DeLoid *et al* determined a simple method to measure the effective density by means of a Volumetric Centrifugation Method (VCM) (DeLoid *et al.*, 2014). This method is based upon centrifugation of a sample at low speed in a packed cell volume tube, and measurements were found to agree with the more costly Analytical Ultracentrifugation (AUC) method (Carney *et al.*, 2011; DeLoid *et al.*, 2014). Agglomerates are important in terms of sedimentation (and diffusion) as the sedimentation speed increases proportionally to the square of the particle diameter and is proportional to the difference between the medium density and the particle effective density (Cui *et al.*, 2016). The agglomeration rate, and therefore sedimentation rate, can be modulated by changing the balance of attractive or repulsive forces between NMs, which can be achieved through surface modification, or by altering the properties of the media (pH, ionic strength, ionic composition) (Keller *et al.*, 2010). Increasing the stability of NM

suspensions, either by electrostatic or steric stabilization, decreases the agglomeration rate. An example of this is the presence of proteins within the medium (such as BSA) which can stabilize NM suspensions, presumably due to steric stabilization, leading to a reduction in the agglomeration rate and therefore changing the sedimentation rate of the NMs (Allouni *et al.*, 2009).

Agglomeration changes the size, shape and effective particle density thereby modulating transport time. The effective density of agglomerates will also vary considerably compared to that of the primary NM density, due to the inclusion of media within the agglomerate. As an example of why changes to NM density are important, buoyant NMs with low density compared to the media, can float and rise away from cells over time, rendering dose response relationships difficult or even impossible to ascertain (DeLoid *et al.*, 2014; DeLoid *et al.*, 2017). This clearly has an implication for study design, i.e. use of standard (submerged), or inverted cell culture systems (see Figure 3). If parameters such as NM size, effective density, viscosity of the medium, along with agglomeration potential are known, then the rate of gravitational settling can be calculated, along with the time that the NMs will likely take to reach the bottom of the container (or reach the cells at the bottom of a petri-dish).

The shape of the NM will also lead to changes in drag and buoyancy forces acting upon the NM, thereby modifying the transport time, and is suggested to lead to an increased sedimentation under the exposure conditions. Shape will also affect the agglomeration and aggregation potential of the suspension, in addition to agglomeration affecting the particle 'shape' (Alkilany and Murphy, 2010). However, it has been suggested that NMs with an aspect ratio of less than 2 can be adequately represented as spheres, while those that have an aspect ratio greater than 2 require alternative models (Herzhaft and Guazzelli, 1999; Teeguarden *et al.*, 2007b; Swaminathan *et al.*, 2012). The effect of shape on sedimentation has been reported to be very different between static and dynamic systems under flow, which is an important consideration for *in vivo* translation since most *in vitro* models are static (Bjo *et al.*, 2016) However, perfusion approaches are available and are typically used to mimic interaction of NMs with blood vessels (Tian and Finehout, 2008; Prabhakarpandian *et al.*, 2011; Albanese *et al.*, 2013; Rennert *et al.*, 2015; Bjo *et al.*, 2016). As represented in the schematic illustrations in Figures 1, 4, 5 and 6, other parameters will also influence the rate of transport such as the height of the container which has an impact on the time that a NM

will take to settle, as does the material and shape of the container, discussed later in the section 'role of media height and container'.

**Diffusion** is a transport phenomenon that occurs due to the gradient of concentration and is represented by the first term in Equation 1. In the absence of sedimentation Equation 1 reduces to Fick's second law, which describes the concentration changes that occur over time due to diffusion. Starting from a well-mixed solution with constant concentration we expect no net particle transport. However, the concentration of NMs at the boundaries will often change with time – such as during cellular exposure where NM's will be removed due to internalization thus creating a concentration gradient. As a result there will be a diffusive transport of NMs towards the adsorbing surface. The typical transport length that a nanoparticle diffuses over time is given by the mean squared displacement

$$\langle x^2 \rangle = 2Dt \qquad \text{(Equation 3)}$$

where *D* is the diffusion coefficient and *t* is time.

Since the diffusion coefficient for spherical NMs is related to the NM size according to the Stokes-Einstein equation, NM diffusion rates are a function of NM size and viscosity of the medium (effective particle density) (equation 4). The Stokes-Einstein equation describes the relationship between the rate of diffusion (D, $m^2/s$) as a function of viscosity (ŋ and temperature (T, $^oK$):

$$D = \frac{k_b T}{6\pi \eta d} \qquad \text{(Equation 4)}$$

where *R* is the gas constant (L kPa/K/mol), $k_b$ is the boltzmann constany, and *d* the NM diameter.

The diffusion coefficient is inversely proportional to NM size; hence smaller particles diffuse more rapidly than larger ones. Diffusion is therefore the dominant force governing delivery of small NM to cells *in vitro*. Larger particles (or agglomerates/aggregates) are transported more through sedimentation forces (Cohen 2015). Nevertheless, the motion of NMs in fluid

media, as well as the transport of macromolecules and colloidal particles, can be governed partly by diffusion forces. The Brownian motion in NM dispersions gives them high mobility, but also an increase in the probability for collision that can lead to surface interactions and to the formation of agglomerates leading to destabilisation of the suspension. These agglomerates, as previously mentioned, can have very different properties that modify the diffusion rate, such as decreased effective density and decreased surface area to mass ratio (as the structures are not being fully solid due to entrapped liquid between the NMs), increased volume, and different shapes. Likewise, dissolution of NMs will change the size distribution of the suspension; smaller NMs will dissolve at an increased rate, therefore changing the concentration and size distribution of the suspension. Sedimentation and diffusion processes are both a function of medium density and viscosity, and are dependent on NM size, shape, charge, and/or density of the NMs. These phenomena can therefore be competitive or act together on NM transport, and can affect the time that NMs require to come into contact with cells in the dish, and thus to become active at the cell surface.

**Role of the exposure time**

Toxicity experiments are typically performed over 24-48 h, but the NM diameter and density can radically change the time a particle requires to reach the cell surface. Indeed it is reported that the time taken for 90% of a NM dose to be deposited can vary between <10 hours to >200 hours depending on the NM in question, which clearly has big implications for the delivered local dose (Cohen, Teeguarden and Demokritou, 2014). This can alter the number of NMs truly interacting with the cells during the exposure time. Figure 4 shows how particles cross dissimilar distances at a particular time of exposure. For example, while PS NMs less than 3 nm in diameter might be able to travel 5 mm in 24 h, 100 nm PS NMs would require much longer to travel the same distance. If only a few NMs are able to reach the target cell in this time, uptake may be reduced and consequently the true “internalised dose” will also be reduced. An example that can illustrate these problems is that of a NM that agglomerates or aggregates rapidly to form larger structures. These structures will then sediment and deposit more quickly onto the cell surface, and therefore may be internalized at a faster rate than initially predicted based on the primary NM size, due to increased contact. An alternative scenario is that agglomerates and aggregates may be too large to enter cells, leading to a reduction in internalization rate (Hussain *et al.*, 2009).

A small toxic NM may sediment slowly, and therefore over the same administered dose, and the same experimental time course, the contact dose and internalized dose could be substantially lower than in the case of a larger but less inherently toxic NM (DeLoid *et al.*, 2017). As a result, the larger NM suspension may lead to an apparent increase in cellular toxicity due to the higher received dose, and be reported as more toxic than the smaller NM, as the delivered dose was higher (DeLoid *et al.*, 2017). It is worth noting that dose is inevitably not the only cause for increases or decreases in toxicity. Parameters such as shape, surface coating, protein corona, shape and core will all influence toxicity. However, in situations where the delivered dose of NMs differs over the same experimental time course, toxicity assessments can be confounded and comparisons between the NMs cannot be accurately made unless these dose effects are properly measured and accounted for. Experimentally, it was shown that the delivered dose of NMs might be low for particles smaller than 100 nm when diffusion plays a predominant role (L.K. *et al.*, 2005; Teeguarden *et al.*, 2007a; Lison and Huaux, 2011). However, this also depends on the density of the material and for instance we have determined that with polymeric NMs like PS of 50 nm diameter, the transport in the extracellular medium for NM to reach the plasma membrane is not rate-limiting (Salvati *et al.*, 2011). Besides the physicochemical properties of NM and medium, other aspects of the exposure or experimental procedure can influence the time of NM transport, including the container volume, the medium height, stirring, and interactions with biomolecules, as discussed below.

**Role of container and medium height**

The medium height describes the distance that NMs in suspension must travel in order to come into contact with the floor of the vessel (or the cells plated at the bottom of the vessel). Medium height can affect both the sedimentation and diffusion of NMs. As stated previously, diffusion time increases with the square of diffusion distance, thus a linear increase of medium height, regardless of the increase in administered dose, results in a distance squared increase in the diffusion transport time. In particular, this highlights the importance of maintaining consistent media height in and across experiments, as altering this could confound comparisons between different NMs. Likewise, decreasing the volume and subsequently the media height might conversely lead to an increase in the number of

NMs in contact with cells. This could have implications for comparisons between different approaches, for example, toxicity studies performed in 96 well plates versus NM uptake studies imaged by confocal microscopy where the coverslips are cultured in 6 well plates. However, with the correct reporting this can be compensated for by recalculating cellular dose, accounting for well size and volume. In several cases it has been noted that extracellular NM concentration is in large excess compared to uptake levels, thus the extracellular NM reservoir can be considered substantially fixed during the full length of the experiment, provided the exposure time is not on the order of days (Kim *et al.*, 2012). Media height could be a problem particularly in toxicity assessments that take place over long durations, whereby the medium volume will evaporate over time, altering the transport in a time dependant manner. It is therefore critically important to record changes in the medium height over time or to replenish media using continual flow to maintain media height (Bjo *et al.*, 2016). Moreover, in the case of decreased extracellular volume and low concentration of NMs, additional complications due to NM depletion, NM dissolution and subsequent concentration changes following uptake may arise. This implies that it is important to include consideration of extracellular volume in the description of the experiments performed. Likewise, in chronic, continual or repeated exposure scenarios where medium is constantly replenished with new treatment media, more complex methods for monitoring and evaluation of medium height, NM transport and dosing over the period would be necessary. In these types of experiments, sophisticated systems for delivering specifically determined doses or measuring delivered doses would be required to enable accurate calculations of cellular dose over the entire time period, such as those used in a 14 week chronic dosing of AgNPs to skin cells (Comfort *et al.*, 2014). This highlights the critical nature of NM characterization under the correct experimental conditions and time courses – NM dissolution, and NM concentration and/or medium height changes over time should all be properly characterized, along with physicochemical characterizations prior to *in vitro* testing. The size and shape of the container is an important aspect of the settling velocity of NM suspensions, as shown in Figure 5 based on experimentally determined settling dynamics of fibres (Herzhaft and Guazzelli, 1999). Open containers often exhibit meniscus effects and hence the inhomogeneous NM surface distributions. The use of closed containers, such as channels, leads to homogeneous NM distributions.

**Surface effects: meniscus and well**

The type of chamber (open versus channel chambers), the size of the chamber, and the material of the chamber have an additional impact on surface properties, such as roughness and polarity. These surface effects (e.g., surface tension) may change local medium composition or concentration of NMs, thereby influencing the transport processes and time, as mentioned above. In channel chambers or culture wear, NMs may be adsorbed onto the surfaces (heteroagglomeration), changing the number of NMs available to be taken up or interact with the cells; again if some NMs are removed from the system through absorption, particularly if this is size specific as suggested, the transport of the remaining NMs will differ from that of the initial suspension. Moreover, a local increase in concentration in a specific part of the chamber could destabilize the suspension, increasing the likelihood of interactions such as agglomeration, again modulating the transport of the resultant NMs. Experiments could be performed to calculate the recovery from chambers and wells to determine the amount of NM binding in different containers of different materials to account for this in experiments. For metallic NMs, this test of NM binding can be performed by conducting the experiment within the culture vessels to be used but without the presence of cells, and for example NM (and ionic) concentrations could be tested (e.g. by single particle Inductively-coupled plasma mass spectrometry (spICP-MS)) prior to, and after incubation with culturewear to determine the NMs remaining in the media at certain time intervals and the amount lost; this would also provide valuable information regarding the dissolution during the experimental time course (Aznar *et al.*, 2017). For non-metallic NMs, intrinsic fluorescence, Raman or other detection approaches could be applied, but often have lower sensitivities.

**Exposure including convection and stirring**

The presences of agglomerates and aggregates complicate the modelling and analysis of dose-metrics and toxicology data. Both agglomerates and aggregates tend to settle and amass, leaving a portion of the cells with a very low delivered dose. To bypass this problem the delivery of NMs can be made under agitation. Mixing and heating affect the variability of the fluid composition, the Brownian motion, the transport towards the cell surface, and ultimately, the delivered dose. In the presence of convection forces all the NMs, regardless their size and density, might be able to reach the bottom of the container and therefore be

active on the cell surface (Lison and Huaux, 2011). However, stirring or heating of a NM solution can increase the number of collisions, thus increasing the likelihood and frequency of agglomeration. In dispersions where agglomeration occurs, competition between diffusion and sedimentation phenomena are more difficult to predict, however using the equations mentioned earlier, and accounting for these different agglomeration rates, models have been constructed that can mimic this effect when calculating the delivered dose (Hinderliter *et al.*, 2010; DeLoid *et al.*, 2017). Agglomeration and aggregation change NM size and surface area and increase the gravitational settling, in other words the presence of agglomerates and aggregates transforms a dispersion of NMs. Characterization of the stability of a NM suspension, particularly in the relevant media for exposure, is therefore necessary in a nanotoxicity study because, ultimately, the stability (or lack thereof) modifies transport in cell culture medium and affects the time a NM dwells in that environment, and hence also their bioavailability. Stability should therefore be assessed under the exposure conditions for, at a minimum, the experimental time course at appropriate conditions, i.e. 37 °C in the same vessel in which they would be applied to cells. Stability assessments generally include measuring the zeta potential to determine electrostatic or steric stabilization of the NMs, DLS to measure particle agglomeration and stability of the size distribution, and NM solubility in control and test medium, all of which will affect the transport and behaviour of the NM suspension (De Campos *et al.*, 2004; Moore *et al.*, 2015; Nur, Lead and Baalousha, 2015; OECD, 2016; Rossi *et al.*, 2016; Avramescu *et al.*, 2017; Gao and Lowry, 2018).

**NM-protein corona effects: cell media and exposure route**

NM size, shape and surface charge have been described as important determinants of uptake (into cells and tissue) and toxicity (Wilhelm *et al.*, 2003; Alkilany and Murphy, 2010; Huang *et al.*, 2010; Fröhlich, 2012; Huk *et al.*, 2014; Kettler *et al.*, 2014; Bjo *et al.*, 2016). It has also become increasingly apparent that the layer of proteins that adsorb at the NM surface and form the dynamic 'protein corona' play a key role in modulating the interactions between NMs and living cells, in particular regarding targeting and uptake behaviour (Nel *et al.*, 2009; Monopoli *et al.*, 2011, 2012; Lesniak *et al.*, 2012; Salvati *et al.*, 2012). The high reactivity of NM surfaces makes the adsorption of proteins, ions and other molecules in solution easier. As a consequence, as soon as NMs are dispersed in a biological fluid, proteins or other biomolecules immediately adsorb to their surface, changing their physico-chemical characteris-

tics (i.e., size, charge and biological activity). Cell media usually contains serum or other charged biomolecules, which modify the NM surface, forming the so-called NM-protein corona (Cedervall *et al.*, 2007; Klein, 2007; Nel *et al.*, 2009; Monopoli *et al.*, 2011, 2012; Pino *et al.*, 2014; Yallapu *et al.*, 2015). This leads to a new 'biological identity' of the NMs, with different size, charge, and surface properties. These size changes can disturb the Brownian motion and the transport phenomena as previously described, and changes in the NM surface charge can alter the affinity for the cell surface or the interaction with petri dishes and ions in solution, and therefore ultimately the bioavailability. Therefore, corona formation can alter the stability of the NM dispersion and modify the interaction with cells (Lynch, Salvati and Dawson, 2009; Tenzer *et al.*, 2011, 2013; Lesniak *et al.*, 2012; Bertoli *et al.*, 2016). Indeed, in the absence of biomolecules in the cell culture media the high surface reactivity of NMs results in significant cellular damage and the acquisition of a corona from biomolecules pulled out from the cell through membrane damage (Lesniak *et al.*, 2012). A complete understanding of how the properties of NMs and their surroundings modulate the corona formation will aid advancements for NM targeting immensely and will be crucial in understanding and predicting the biological effect and fate of NMs, both from direct exposure (e.g. nanomedicine) and indirect exposure (e.g. via the environment (Lynch *et al.*, 2013, 2014)).

The NM corona constitutes the primary point of interaction between the NMs and biological components and is known as the ‘nano-bio interface’ (Nel *et al.*, 2009). Due to the formation of this protein corona, the presence or absence of serum proteins in cell culture studies has a major impact on subsequent effects (Lesniak *et al.*, 2012; Cheng *et al.*, 2015; Fleischer and Payne, 2015; Ritz *et al.*, 2015; Shannahan, Podila and Brown, 2015). The presence of the protein corona can modulate cellular internalization patterns, and lead to increases or decreases in NM uptake a cell specific manner. In several cases it has been reported that uptake of NMs in the absence of proteins (serum free conditions) is much higher than the uptake observed in the presence of serum (such as FBS), likely due to a reduction in non-specific binding of the NM at the cell surface in comparison to that observed in the absence of proteins (Lesniak *et al.*, 2012; Smith *et al.*, 2012b). This is supported by the observed increase in the binding of NM to the cell surface in the absence of proteins, indicating that interactions between the membrane and the bare NM lead to adhesion at the cell surface and increased likelihood for internalization (Smith *et al.*, 2012b; Lesniak *et al.*, 2013). Lesniak

et al also demonstrated that $SiO_2$ NMs exposed under serum free conditions had acquired a protein corona within 1 hour of exposure, by pulling proteins from the cell membrane (Lesniak *et al.*, 2012). It is suggested that the NM protein corona, in a static environment, can reach steady state in minutes to hours (Albanese *et al.*, 2014). Albanese et al, reported the evolution of the corona following the release of proteins and nutrients from the cells (in a process they term conditioning) (Albanese *et al.*, 2014). Some of these secreted proteins may have a higher affinity for the NM surface than the previously bound proteins, leading to exchange and evolution of the corona during the entire exposure time (Albanese *et al.*, 2014). Albanese *et al* determined an optimum NM incubation time of 4 hrs in the conditioned media prior to protein corona characterization, but postulate that time-dependant changes *in vivo* are likely to be far less remarkable than the changes that occur due to translocation through blood and tissues (Albanese *et al.*, 2014). Based on their findings, corona characterization should be performed in both unconditioned and cell conditioned media to give the best indication of the biocorona evolution.

Based on the above discussion, it becomes evident that experiments that are performed in the absence of proteins or other biomolecules are not biologically relevant as bare, uncoated NMs will not be present in biological fluids *in vivo,* as protein and biomolecule adsorption occurs almost instantaneously, and indeed even under serum free conditions the NMs rapidly pull proteins from the cells to form a corona (Lesniak *et al.*, 2012). In this context, the use of different concentrations of FBS may also need to be investigated in terms of corona composition and evolution. It is known in fact that the corona formed on the same NM changes when different protein concentrations are used, for instance to resemble more closely the protein concentration present *in vivo* in human serum, as opposed to the more diluted serum concentrations typically used in *in vitro* studies (Monopoli *et al.*, 2011). The concentration of proteins of the media not only changes the composition of the corona but also affects the overall uptake levels into cells, thus the effective dose delivered (Kim *et al.*, 2014). Another important factor is the source of the serum proteins. Individual batches can vary in composition and quantity of bioactive compounds, therefore centralized batches of serum are favoured for large scale NM studies and new emerging serum free alternatives are an area of interest (Baker, 2016). Indeed centralized batches of serum are commonly used in large interlaboratory comparisons such as those organised within QualityNano and other

similar efforts (see Salvati *et al*, in this special edition). Thus, the exposure conditions should be fully specified in terms of composition of the extracellular medium, and the composition of the corona itself as thoroughly as possible.

## Uptake of nanoparticles into cells

Different cell types are designed to carry out very different specific functions *in vivo*; this leads to varied cell surface protein / receptor expression, internalization mechanisms and detoxification processes (Kuhn *et al.*, 2014a). The extent and fate of cellular accumulation can depend largely upon the internalization mechanism utilized and the properties of not only the NMs, but also the cells themselves (Kettler *et al.*, 2014). It has recently emerged that the proteins bound at the surface of the NMs (such as those present in the corona) can be recognized by cell surface receptors, leading to NM internalization and trafficking via the endo-lysosomal system (Bertoli *et al.*, 2016; Lara *et al.*, 2017). Coating with serum proteins thus confers specificity to NM internalization pathways upon binding to a membrane receptor, an example is that of the transferrin receptor (TfR), whereby transferrin presence in the protein corona leads to the subsequent internalization through TfR interaction and clathrin mediated endocytosis (Figure 2) (Mazzolini *et al.*, 2016). In some cases however binding and recognition of a certain corona protein may activate internalization via pathways that differ from that usually triggered by that protein (Mahon *et al.*, 2012). Protein coated NMs could therefore be internalized preferentially by different uptake machinery, dependent upon the proteins present in the NM corona, and pathways active within a cell or cell population, and can therefore greatly influence cell uptake, fate, localization and toxicity (Aggarwal *et al.*, 2009; Lesniak *et al.*, 2010, 2012; Oberdörster, 2010; Mortensen *et al.*, 2013; Treuel *et al.*, 2014; Wolfram *et al.*, 2014; Grafe *et al.*, 2015; Bertoli *et al.*, 2016; Mazzolini *et al.*, 2016). It has been suggested that the presence of apolipoproteins, such as Apo E or Apo H, in the corona of NMs could lead to higher uptake efficiency, and indeed Apo E and ApoB-100 are also known to support the transport of NMs across the Blood Brain Barrier (BBB) (Kreuter *et al.*, 2002; Wagner *et al.*, 2012; Wolfram *et al.*, 2014; Ritz *et al.*, 2015; Neves *et al.*, 2017). More recently it was demonstrated that overexpression of specific cell receptors, such as the transferrin receptor, low density lipoprotein receptor and Fc-gamma receptor 1, can lead to increases in NM accumulation (Mazzolini *et al.*, 2016; Lara *et al.*, 2017). The interactions between NMs and proteins is therefore crucial to study, both in terms of determining NM up-

take patterns, but also to investigate any conformational changes that occur to proteins due to the NM binding, which can affect their receptor-binding efficiency. Approaches to achieve this are beyond the scope of this review, but a short discussion on this is included in the Supplementary Information.

This receptor mediated internalization can lead to subsequent degradation and release of NM metabolism products within the cell, such as free ions and components of the NM coatings, leading to toxic responses such as generation of ROS and DNA damage (Arbab *et al.*, 2005; Lara *et al.*, 2017). NMs have been found to utilize a variety of mechanisms to enter different cell types, including membrane permeabilization and dynamin dependant pathways, clathrin mediated and caveolae mediated endocytosis or clathrin-independent endocytosis (Rejman *et al.*, 2004; Yang *et al.*, 2007; Dausend *et al.*, 2008; Smith *et al.*, 2012a; Zhu *et al.*, 2013; Mazzolini *et al.*, 2016). However, there is still no clear factor that determines which route is preferentially employed and the detailed mechanisms NMs use to enter cells still remain unclear (Iversen, Skotland and Sandvig, 2011). A variety of methods are available to investigate this specific uptake including drug and siRNA inhibition studies, fluorescence confocal colocalization studies and reflectance imaging (Daldrup-Link *et al.*, 2003; Matuszewski *et al.*, 2005; Stefaan J. H. Soenen *et al.*, 2010; dos Santos *et al.*, 2011; Sandin *et al.*, 2012; Kuhn *et al.*, 2014b; Guggenheim *et al.*, 2016). These different approaches have various advantages and limitations: for instance fluorescence studies require fluorescently detectable NMs, reflectance methods require optically dense NMs, colocalization studies are limited by the resolution of the imaging system and the quantification methods employed, and drug inhibition of cellular pathways can lead to cell-wide cytotoxicity (Bolte and Cordelieres, 2006; Stefaan J H Soenen *et al.*, 2010; Iversen, Skotland and Sandvig, 2011; Pike *et al.*, 2017). New emerging combinations of techniques can circumvent some of these problems, such as the use of correlative reflectance and Transmission Electron Microscopy (TEM) investigations. In these investigations, light microscopy (LM, confocal or superresolution) can be employed to measure the effects of specifically inhibited pathways alongside the visualization of labelled compartments and cellular features in fixed or living cells. Ultra-high resolution can also be maintained using Electron Microscopy to visualise individual NMs and the cellular ultrastructure (Guggenheim *et al.*, 2016). This permits identification of subcellular localization and nature of signal, colocalization of NMs with labelled components and the visualization of the

cellular ultrastructure. Correlating these methods also allows the visualization of dynamic events using LM, followed by high resolution investigation at a fixed point within the dynamic process providing valuable information that may otherwise have been missed (Karreman *et al.*, 2016).

Despite the idea that cellular dose, and subsequent NM internalization, is largely responsible for cell cytotoxicity, it is not the only potential source of deleterious effects. NMs have been suggested to elicit cell signalling responses that can lead to subsequent cellular stress via paracrine methods, such as inducing the release of proteins such as cytokines (Raghnaill *et al.*, 2014). In this way, NMs can affect other cells without the need for actual physical interaction. Cobalt chromium NM exposures on one side of a multi-layered barrier, for example, have been found to lead to cytotoxic effects such as DNA damage and chromosome aberration to unexposed cells on the other side of this barrier without actually crossing (Sood *et al.*, 2011). Another study indicated that the interaction of carboxylate modified polystyrene (PS-COOH) NMs with the BBB, for example, led to changes in the levels of pro-inflammatory and pro-survival cytokines, such as RANTES, TNFR1 and EGF, released in the presence of glial cells with which they were not in physical contact (Raghnaill *et al.*, 2014). These subtle effects of NMs would largely be missed by the traditional acute toxicity testing strategies, and an important consideration when making toxicological assessments of NM is their accumulation potential, subsequent cellular stress and the potential for effects on paracrine signalling between cell types.

## Modes of NM exposure in cell culture

This section discusses the existing approaches to present NMs to cells and how they translate into requirements SOPs.

### Upright exposure of NMs to adherent cells

The most common mode of NM exposure for standard submerged adherent cell cultures is the addition of NMs in the extracellular medium as depicted in Figure 1. Still, even in this standard configuration, the ways NM dispersions are prepared and added can be multiple and this can lead to different results. To illustrate this, experimental data provided in five different articles which studied $TiO_2$ NM interactions with adherent cells in an upright

configuration, were assessed. The data are summarised in Table 1. In each case it was stated that the NM dispersion was diluted into cell culture medium following sonication. The duration of the sonication step varies extremely between publications, as shown in Table 1. The DSE was not calculated or optimized in any case, which has previously shown to be of importance, however in three of the cases the sonication energy was provided (DeLoid *et al.*, 2017). Furthermore, the inclusion of FBS differed between the studies, 3 included FBS, 1 was omitted FBS and 1 did not state whether FBS was present or not. The effect of the inclusion or omission of FBS in cell studies has been studies in depth, as previously discussed. After addition to cell medium, the NM suspensions were characterized in all cases by at least one technique. However, it is important to use at least two size characterization methods, due to the necessity to measure core and hydrodynamic size following immersion in different fluids; different techniques measure different size end-points. In one case only the DLS size was reported, giving no information regarding the result quantified from the TEM measurements nor the nominal size of the NM. Different multi-well plates were used in all studies, which as discussed, would lead to changes in the delivery and quantity of the NM dose, but if correct parameters were recorded, could be accounted for and allow comparison across the studies. In each case the size of the well was recorded, however variations exist between manufacturers, which will lead to small changes in the total surface area and therefore the manufacturer should also be reported to enable accurate calculations. The incubation time was reported as 24 hrs, independent of the height of the media and the size of the NMs, therefore the dose delivered to the cells would have been different in each case – and was not calculated, rendering comparisons impossible. Additionally, the total volume of NM suspension added per well was given in only one of the five publications assessed (see Table 1). Consequently, parameters such as the height of the liquid column (which will affect the diffusion and sedimentation of the NM) or the total amount of NMs added to the cell culture remains unknown in the cases where the media height is not given, therefore the cellular dose cannot accurately be calculated. There are currently no standard units for the recording of NM concentration in solution, which is often given in µg/mL (4/5 cases) or µg/cm$^2$ (2/5 cases) – in one case it was given in both. The cell seeding density, cell density at exposure and cell size should also be recorded, to allow accurate calculations of NM dose per cell (where all other parameters are recorded). Inclusion within SOPs of automated techniques that calculate the number of cells in a microscopical field of view, or by means or alternative

cell counting methods, to extrapolate to estimates of cell number within the dish would be of benefit here, a technique validated previously (Jaccard *et al.*, 2014). If appropriate measurements of these aforementioned experimental metadata are recorded, then cellular dose can be calculated (following experimental determination of the effective densities and ENM dissolution) using models such as the ISSD or the more recent Distorted Grid model (Hinderliter *et al* 2010; DeLoid, 2017). Ideally, the delivered cellular dose *in vitro* (and *in vivo)* would be measured directly, using techniques such as spICP-MS, however the cost and/or availability of such techniques in biology labs is often limited.

Within the QualityNano research infrastructure, extensive work was performed to develop standardized protocols for different assays in nanosafety testing, such as to quantify nanoparticle uptake by flow cytometry and to measure nanoparticle-induced cytotoxicity (Salvati *et al.*, 2017). In order to minimize interlaboratory variability that could generate from all of the aspects discussed above, the developed SOPs also included details on how to perform cell seeding, NM dispersion preparation in cell culture medium and exposure to cells. Prior to further testing on cells, participating laboratories were asked to demonstrate their proficiency in cell culture, following the SOPs for cell culturing and for assessment of cell growth (thee SOPs are included in Nelissen et al. 2017). For these tests, lung cancer epithelial A549 cells were selected as a common model cell line easy to maintain and often used in nanosafety testing. In order to limit the variability arising from differences in cell handling and storage procedures, frozen cells amplified from the same initial cell stock at the same passage number were provided to all laboratories. Similarly, FBS from a common stock was distributed. Different serum batches are known to have different protein composition and concentration as mentioned previously: this can affect cell growth and proliferation rate. Additionally, when serum is used to prepare NM dispersions, it can lead to differences in NM stability and the corona composition, thus ultimately leading to different behaviour in cells. NM dispersions in water were prepared from a common stock and aliquots shipped to all laboratories. Reporting forms were developed to record all details and timing of cell culture medium preparation, aliquotting of the received FBS stock, cell defrosting and sub-culturing. Further details of the quality control procedures and process are provided in the Supplementary Information. The NM uptake SOP and NM-induced cytotoxicity SOPs contained details on how to prepare the cell dilution for cell seeding and the volumes of the

diluted cells to be added to each well for the different multi-well plates used. Additional precautions were taken to limit clumping of the cells to the periphery of the wells due to edge effects and obtain a more uniform cell density throughout the well (see Nelissen et al. for details). NMs known to form uniform and stable dispersions in cell culture media containing FBS, such as PS-COOH NMs and amino-modified PS (PS-$NH_2$) were specifically selected for these interlaboratory comparisons. For these PS NMs, simple vortexing ensures preparation of good dispersions (as mentioned above other particles are known to require much more complex dispersion protocols). Even though the procedure is relatively simple, the SOPs contained details on the volumes added, order of mixing, pipetting strategy (forward pipetting etc.) speed and time of vortexing and timing between preparation and addition to cells, all factors that could affect the final outcome. Additionally, variability due to NM preparation was estimated by preparing 3 separate NM dispersions, each representing a separate technical replicate. A separate dosing plate was used to prepare serial dilutions from 3 freshly-made NM dispersions in cell culture media at the highest concentration tested. Finally, cells were exposed to the NM dispersions by replacement of the extracellular media with specified volumes of the freshly-made NM dispersions from the dosing plate. Addition of small volumes of NMs at higher concentration directly into the wells containing cell culture medium can be more difficult to control and can lead to strong differences in exposure for the different cells within the well. Overall, by taking into account all of these details, the cell growth proficiency test, NM uptake studies by flow cytometry and NM-induced cytotoxicity assays, all gave highly reproducible results in independent laboratories (see Nelissen et al and Salvati et al for details). It is clear that this kind of SOP captures many more details and instructions compared to what is typically reported in the experimental sections of published articles (e.g. 8 page protocols, as indicated in Nelissen et al and Salvati et al).

**Other modes of NM exposure to cells**

As discussed previously, NM consisting of various materials and with different sizes and shapes show different sedimentation and diffusion rates, leading to different cellular doses of NM over time. NMs are heterogeneously distributed to the cells particularly in the standard upright configuration whereby NM dispersions are placed over cells (Figure 3).

Other techniques exist for cellular exposure of NMs, including surface based, whereby NM are decorated onto a solid surface, inverted culture and air-liquid interface (ALI). Figure 3 presented an overview of the different modes of presentation available, and here these are described in more detail, and related to the parameters required as part of the SOP to ensure comparability with other studies and the classical submerged culture approach (see Table 2 and section 'Recommended Reporting' and appendix 'Protocol').

**Surface based presentation of NMs to cells**

In the surface based presentation of NMs to cells, NMs are immobilized onto the surface prior to cell exposure and cells seeded on top of the NM layer, as shown schematically in Figure 3 (Alberola and Rädler, 2009). This circumvents some of the problems associated with upright culture treatment, as the NM are static, and thus do not undergo agglomeration. A defined number of attached NMs on the surface can be obtained by tuning the NM absorption time and the NM concentration in solution. Figure 6 shows an example of homogeneous Quantum Dot (QD) distributions achieved by varying the NM concentrations in solution (Alberola and Rädler, 2009). This prevents fluctuations in the number density throughout the surface which is important in minimizing the cell to cell dose variability when evaluating cell response in toxicity assays (Snijder and Pelkmans, 2011). Moreover, it enables the precise definition of the NM dose to which the cells are exposed.

This type of set-up confers some advantages; the exact number of NMs/$\mu m^2$ is known and pre-defined and the problems associated with NM agglomeration / aggregation and inhomogeneous distribution over time are circumvented (Alberola and Rädler, 2009). The monitoring of the NM uptake process is also simplified because the NMs, which are initially absorbed on the two dimensional reference surfaces ($\mu g/cm^2$), are lifted above the reference plane when they are taken up by the cells. Uptake can therefore be assessed by evaluating the position of the NMs in a stack of z-scans from confocal microscopy.

In the surface based NM presentation, the interactions between the NMs and the substrate play an important role which should not be neglected. This interaction must be sufficiently strong for the NMs to stay in place after rinsing and the addition of cell medium. However, strong interactions of NMs with the surface can shield the NM-cell interaction and thus likely modulate the internalization efficiency of these systems. For example, when carboxyl func-

tionalized QDs where bound to a poly-L-lysine surface, the cellular uptake was drastically reduced in comparison with an uncoated substrate (Alberola and Rädler, 2009). These systems indicate active uptake processes must be involved due to the uptake against gravitation and surface attraction forces (Alberola and Rädler, 2009). However, the effect that the surface binding of NM to the substrate prior to cellular uptake has on the internalization mechanism should also be carefully considered and evaluated. This configuration also provides a means to investigate the gain and loss of NM over time via endo and exocytosis, as a set number of NMs are present within the system. Surface based presentation-type configurations could be useful for mimicking particular *in vivo* conditions, whereby NMs are static within dynamic conditions (such as QCM measurements for NM/receptor binding). NM could also be embedded within or exposed to an extracellular matrix and presented to cells via or following ECM-interactions. NMs are known to interact with many ECM components, and NM diffusion through ECM has been studied previously (Sykes *et al.*, 2016; Engin *et al.*, 2017). These NM-ECM interactions are undoubtedly going to have an effect on the cellular uptake (and the properties of the surrounding ECM itself) which may be important for the translation of potential cancer therapeutic or diagnostic agents for *in vivo* success as delivery vehicles (Sykes *et al.*, 2016; Millard *et al.*, 2017).

**Inverted cell culture systems**

Inverted cell culture systems have been suggested as particularly well-suited for buoyant NM exposure conditions (Figure 3) (Spyrogianni *et al.*, 2016). In an inverted system, the cells are attached at a coverslip and suspended into the media from above. The use of inverted systems can be preferential when the NMs have a low effective density compared to the medium, these systems have therefore been compared to the use of standard upright exposure conditions (Watson *et al.*, 2016). Polypropylene (buoyant) NMs led to cellular toxicity only in the inverted configuration, and not in the upright configuration, indicating limitations of current systems that rely solely on the upright configuration (Watson *et al.*, 2016). One study compared the effects of soluble and insoluble NMs in both configurations, whereby it was observed that dissolvable NMs showed no difference in the toxicity using either technique, when it was the ion itself known to cause the toxicity (Spyrogianni *et al.*, 2016). However insoluble particles exhibited different sedimentation rates and therefore deposited doses, leading to observable differences in cytotoxicity (Spyrogianni *et al.*, 2016).

In upright configurations NM will come into contact with cells through both diffusion and sedimentation, where in inverted configurations NM exposure to cells will be through diffusion only, and sedimentation will lead to loss of material and lower available NM number. One study compared this effect using different size and composition NMs, all of those larger than 15 nm but smaller than 118 nm showed greater uptake in the upright configuration, but differences were more prominent in the larger particles (Lison and Huaux, 2011). Smaller particles, less than 15 nm showed similar uptake in both arrangements, presumably due to their predominant diffusional movement, those larger than 118 nm had larger sedimentation forces and showed much greater uptake in the upright configuration (Lison and Huaux, 2011). This work highlights how culture conditions can modify the delivered dose in a significant way and has important implications for toxicity studies.

Another approach to control NM exposure to cells could be to first let the NMs adhere on the cell surface in conditions in which uptake is inhibited (for instance by energy depletion at lowered temperature – 4 ºC) and then let the adhered NM enter cells after the extracellular NMs are removed. Such an approach, although far from real biological conditions, has been applied in order to determine the role of the initial adhesion to the cell membrane in the overall uptake level and to explain the observed differences in NM uptake in the presence or absence of a corona on the NM surface (Lesniak *et al.*, 2013). The comparison of the uptake achieved after increasing the incubation temperature of the NMs that had previously had lower temperature, with the uptake measured after continuous exposure in physiological conditions (at 37 ºC) determined that the initial adhesion to the cell membrane is a very fast and crucial step in NM internalisation and that it is strongly affected by the presence or absence of proteins on the NMs.

**Air-liquid interface *in vitro***

Despite the existence of the aforementioned exposure methods, including upright, inverted and substrate attached NM configurations; these can still not accurately represent some physiological exposure conditions. Adverse effects that can occur due to inhalation of NMs are an important consideration, and traditional *in vivo* inhalation studies have several associated drawbacks in regards to the use of animals, need for facilities, cost and poor translation of effects (Clippinger *et al.*, 2016). Therefore considerable effort has been

undertaken to mimic relevant exposure conditions in the lung in a method termed air liquid interface exposure (ALI) whereby an automated station can expose cell cultures directly to airborne or aerosolized substances such as NMs and fibres (Hedwig M Braakhuis *et al.*, 2015; Clippinger *et al.*, 2016; Latvala *et al.*, 2016; Mülhopt *et al.*, 2016; Polk *et al.*, 2016; Geiser *et al.*, 2017). In the ALI exposure, cells are plated in trans-well formation, with the media below, and the exposed cell surface available for interaction with the applied aerosols (Figure 3) (Polk *et al.*, 2016). This type of culture system has several advantages, particularly for the assessment of airborne pollutants and aerosolized NMs. The exposure conditions mimic lung inhalation studies, and the delivery of the gas phase NMs circumvents a variety of problems associated with standard cell culture techniques. The NM delivery is dynamic and controlled through an inlet at the top of the chamber, and these NM can be tested directly without changing the properties (e.g. by dispersion in serum containing medium), in addition to bypassing agglomeration effects and the effects of NM delivery within a bolus form, such as that used in standard culture systems. As such, there is now a range of commercially available ALI devices, including ALICE, NACIVT, MINUCELL, VITROCELL and CULTREX systems (Bakand, 2016). ALI devices have been demonstrated to represent a valid model for the toxicological assessment of poorly soluble NMs, including MWCNTs (Chortarea *et al.*, 2015; Clippinger *et al.*, 2016; Loret *et al.*, 2016; Polk *et al.*, 2016). MWCNTs often have high aspect ratios and their low solubility have raised concerns that long term exposure could lead to lung pathologies seen previously with other fibrous particles (Oberdörster, 2010). Therefore efforts are being made to investigate the effects of such NMs using ALI methods (Endes *et al.*, 2014; Chortarea *et al.*, 2015; Polk *et al.*, 2016; Beyeler *et al.*, 2017). A study using one such ALI method was combined with a 3D *in vitro* model of the epithelial airway barrier, combining both sophisticated delivery and a multicellular 3D physiological cellular environment to investigate these high aspect ratio CNTs (Endes *et al.*, 2014; Chortarea *et al.*, 2015). Clearly the combination of physiologically relevant exposure and physiologically relevant cellular environments can provide new insight into the potential toxicity of NMs. The effect of chronic to acute exposure to CNTs were assessed and compared using this methodology indicating changes in the levels of the antioxidant glutathione (Endes *et al.*, 2014; Chortarea *et al.*, 2015).

These ALI devices have been used in the evaluation of early cytotoxicity to cells following exposure to airborne NMs, and pollutants resulting from diesel and gasoline engines (Herzog *et al.*, 2013; Lenz *et al.*, 2013; Mülhopt *et al.*, 2016; Kooter *et al.*, 2017). In the cases of pollutants, the toxicity of the gas phase NM, compared to the removed NM which were subsequently re-suspended in upright culture was markedly increased, indicating the value of this technique in such studies, and the necessity to use the right cell exposure configuration (Mülhopt *et al.*, 2016). ALI systems have also showed significant value in the assessment of aerosols generated by engineered NMs that were previously unattainable using standard culture configurations, such as in the case for zinc oxides, copper oxide and nickel oxides where levels of cellular toxicity were observed at much lower doses in ALI systems compared to standard culture (Holder *et al.*, 2008; Lenz *et al.*, 2013; Kooter *et al.*, 2017). ALI exposure of 3D epithelial co-culture models to AgNPs also provided insight into the lack of toxicity of these NPs at realistic exposure concentrations (Herzog *et al.*, 2013). In these systems the number of NMs deposited at the ALI corresponds closely to the modelled number concentration expected to be observed in tracheal-bronchial regions of the lung; however smaller particles are not deposited as efficiently as larger particles so optimization is sometimes necessary (Holder *et al.*, 2008).

Clearly there are a vast amount of considerations in terms of the different ways in which to dose cells with NMs. *In vitro* toxicity assays should be designed such that they can ultimately predict toxicity *in vivo* and thus replace, at least in part, *in vivo* assays in-line with the 3R framework (Fischer and Chan, 2007; Stone, Johnston and Schins, 2009; Burden *et al.*, 2017). In terms of deciding upon the culture configuration to use, the question that naturally arises is which conditions are more representative of the *in vivo* exposure being assessed. Naturally, this depends on the system, exposure route and process to be modelled. If the purpose is to study NM cellular uptake after inhalation, none of the upright, surface nor inverted approach is adequate, since the epithelial cells in the lung are polarized and the NMs would be exposed to them from the basal side instead of the apical side, as happens in natural conditions. Therefore, the only appropriate method is using ALI whereby the apical side is exposed to aerosol as it would be *in vivo.* For studies within cells that do not show polarization, such as fibroblasts, the location of the NMs may be less crucial. If the aim is to study how NMs are eliminated from the body, travelling from the blood stream towards the

lumen of an organ, the surface based presentation would allow observed uptake from the basal membrane of epithelial cells. For example, it has been shown that the uptake rate of fluid-phase markers over the basal side was six times higher than for the apical side due to the bigger area of basolateral surface domains (von Bonsdorff, Fuller and Simons, 1985). In a more physiologically accurate method, microfluidic devices allow for the introduction of cellular perfusion in single or multicellular 2D and 3D structures, these are discussed more later in the section 'NM uptake and transport under more physiological conditions' (Tian and Finehout, 2008; Prabhakarpandian *et al.*, 2011; Rennert *et al.*, 2015).

Nevertheless, regardless of which configuration is used there is a minimum requirement for information and standardization of these procedures, of which there has been considerable debate. Several of these have been discussed or introduced in the previous sections. Recordable attributes include experimental parameters such as the cell type and exposure configuration used, cell seeding density, the density of cells at time of exposure, exposure duration, exposure media volume and culturewear used (with justifications for aforementioned chosen parameters where appropriate). Additionally, during suspension preparation (if applicable), determination of the DSE, optimization of the sonication step (both time and crDSE), and adequate characterization of the suspension properties should also be performed. The recoded dose should be standardized, but the important aspects include the concentration in terms of NM number and mass per surface area (NM/cm$^2$), and any conditions that may modify the locally received dose. Modelling of the agglomeration and transport where possible would also be advantageous. Development of Graphical User Interfaces (GUIs) that can aid in determination of the local dose would be ideal, whereby experimental parameters are input, and a local dose estimated based upon these parameters and the suspension properties, for which a few different models already exist (Cohen *et al.*, 2008; Hinderliter *et al.*, 2010; DeLoid *et al.*, 2017).

## Other factors that can affect NM dose and toxicity

### Role of cell cycle and cell division on NM uptake

Typical *in vitro* experiments are performed in cell populations in which individual cells progress independently through different phases of the cell cycle. In such conditions,

continuous exposure to NM leads first to linear uptake into cells, then at later stages the internalised NM load plateaus as an apparent saturation occurs (Salvati *et al.*, 2011; Shapero *et al.*, 2011; Kim *et al.*, 2012).This saturation effect could be attributed to the NM depletion in the extracellular medium, saturation of cell / cell surface receptors, or competition between uptake and export or degradation of the internalised NMs, in addition to the effect of the cell division. When the cells divide the internalised NM burden is split among daughter cells, (Summers *et al.*, 2011, 2013; Kim *et al.*, 2012). It is therefore important in this context to develop methods that are able to discriminate between these possibilities and to distinguish eventual NM export or degradation from simple dilution by cell division. As a consequence of cell division, after hours of uptake, individual cells within the same population may have varied amounts of internalised NMs (Kim *et al.*, 2012; Summers *et al.*, 2013). Therefore, the internal dose is different and consequently different effects may be observed at single cell level in comparison to the average behaviour of the full population.

Due to this heterogeneity, it has been suggested that the measurement of mean population response is somewhat inadequate for assessment of subtle cellular changes following treatment, such as NM internalisation (Manshian *et al.*, 2015). Neighbouring cells have been seen to undergo different responses to NMs and cell averaging may mask subtle changes that occur in minority populations, an effect that is applicable across most fields of biology. Manshian *et al* showed the value of binning data to determine significant toxicity values in subsets of the population, rather than simply averaging cell values when estimating the safe concentrations of NMs (Manshian *et al.*, 2015). They showed that sub-populations of cells undergo different effects following NM treatments, including a portion that undergo cyto-protective effects and some that undergo cytotoxic effects. This was masked by a net-zero overall change in the cell population measured as a whole.

This all suggests that for quantitative uptake and toxicity studies the initial cell density and the effect of cell cycle should also be taken into account, as different starting conditions will lead to different distributions of the population among the individual phases of the cell cycle and this will lead ultimately to different uptake levels. On another level, it may be important in future to develop methods to distinguish and determine single cell uptake and NM impact in respect to full population behaviours. Other approaches that have been developed are

the use of short pulses of exposure (10 minutes to 1-4 hrs), followed by longer observation times, in order to track movement of NMs inside cells, and to correlate localisation with observed effects (Wang, Bexiga, *et al.*, 2013; Wang, Yu, *et al.*, 2013; Guggenheim *et al.*, 2016). While an analysis of methods for quantification of internalised NMs was beyond the scope, a summary of approaches is provided in the Supplementary Information.

**The bystander effect in toxicity studies**

Another factor to take into consideration is that of indirect effects due to the presence of NMs. Many efforts so far have been focused in controlling NM uptake and defining the dose delivered intracellularly in order to obtain quantitative and reproducible accumulation and correlate the internalised dose with the eventual impacts observed. However, several studies report that NMs can also induce indirect effects by activation of signalling pathways which can propagate to neighbouring cells, even if these cells are not directly in contact with the NMs or indeed regardless of NM internalisation. NMs have been observed to confer the capacity to activate signalling pathways by the interaction of corona proteins with specific receptors at the cell surface or as a consequence of opsonisation and immune system activation (Fadeel and Garcia-Bennett, 2010; Marano *et al.*, 2011; Boraschi, Costantino and Italiani, 2012; Ilinskaya and Dobrovolskaia, 2016; Pallardy, Turbica and Biola-Vidamment, 2017). Such an effect has been reported, for instance, upon adsorption of fibrinogen to 5nm acetic acid capped gold NMs which led to the unfolding of fibrinogen and caused exposure of a cryptic epitope which interacts with MAC-1 receptor on macrophages, leading to activation of NFkB pathway (Deng *et al.*, 2012; Marucco *et al.*, 2014; Corbo *et al.*, 2016). Another example is the changes in conformation of the cytoskeletal protein tubulin in the presence of $TiO_2$ NMs; tubulin carries out a host of different functions within cells and its conformational loss could therefore lead to wide scale disruption of cellular function (Gheshlaghi *et al.*, 2008).

The signalling properties of NMs are also relevant to signalling across cell barriers. For instance, regardless of the capacity of a NM to physically cross cell barriers such as the BBB or the placenta, it has been observed that NMs can induce impact across these barriers in a paracrine fashion (Sood *et al.*, 2011; Raghnaill *et al.*, 2014) (Raghnaill *et al.*, 2014). For example, paracrine signalling has been observed in the case of *in vitro* human blood brain

barrier (BBB) models exposed to NMs in the presence of astrocytes in the basal chamber (Raghnaill *et al.*, 2014). BBB endothelial cells exposed to PS NMs produced relatively low levels of cytokines; however, when astrocytes were added, anti-inflammatory signals were amplified. This also suggests that overall the impact observed in single cell culture systems may be different to that observed in co-culture models or in the presence of multiple cell types, where signalling across the different cell types can amplify and modulate the response due to exposure to NMs, leading to different outcomes. All of these effects should be taken into consideration when assessing NM impact on cell functions.

## NM uptake under more physiological conditions

Efforts to improve the physiological relevance of 2D culture, such as the ALI approach described above, can be further enhanced through, for example, direct measurement of the ALI delivered cellular dose which is possible by the addition of a Quartz Crystal Microbalance (QCM) within the system, underneath the exposed culture in mass deposited per area ng-µg/cm$^2$. This circumvents problems of modelling NM dose (such as in other exposure configurations), as the actual deposited dose is measured. This therefore provides experimental detail of delivered dose, which as discussed, is a necessity for meaningful comparison of toxicological study data which is important in all exposure scenarios. Models of other exposure scenarios (i.e. through ingestion) such as gastrointestinal (GI) tract exist, that aim to either mimic the dynamic changing environment along the entire GI tract, or aim to model the translocation and uptake behaviour in intestinal epithelium models (Hedwig M Braakhuis *et al.*, 2015; Lefebvre *et al.*, 2015). These models consist of simple 2D models (such as the Caco-2 cell line) or more complex multicellular models that contain intestine microfold cells, however these intestinal models lack the mucus layer that is present *in vivo* and *ex vivo* or Glanimal models (Hedwig M Braakhuis *et al.*, 2015; Lefebvre *et al.*, 2015). Although these types of 2D cell cultures clearly offer an invaluable tool for hazard characterization, it is generally recognized that they do not adequately represent the multicellular physiological environment *in vivo (Smalley, Lioni and Herlyn, 2006; Braakhuis et al., 2015; Evans et al., 2017)*. Multicellular functions and cellular interactions are lost when using simplified 2D culture systems which can lead to inaccuracies when assessing potential toxicities and ultimately lead to suboptimal translation to the situation *in vivo* (Smalley, Lioni and Herlyn, 2006; Braakhuis *et al.*, 2015; Henriksen-Lacey, Carregal-Romero and Liz-Marzán, 2016). With

the advent of new approaches (such as those used in IATAs) and multi-parametric and multi-test scoring approaches of NM toxicity, some of the problems can be alleviated (Wiemann *et al.*, 2016; Hansjosten *et al.*, 2017). However, there is an interest in combining the aforementioned *in vitro* exposure models with 3D culture and co-culture models and the use of reconstructed tissues that can incorporate a wide range of physiological conditions more closely mimicking that of native tissue (Evans *et al.*, 2017).

3D cell culture models overcome some of the limitations of traditional 2D cell cultures by the introduction of cell-cell interactions, oxygen and nutrient gradients, and, importantly for nano-research, non-uniform NM dose through the 3D structure (Yoshii *et al.*, 2011; Cui, Hartanto and Zhang, 2017; Evans *et al.*, 2017). Spheroid culture models can be combined with an endothelial cell coating, such as that used by Ho *et al* to mimic the endothelium of blood vessels surrounding the tumour, which can be used to model extravasasion and penetration of NMs (Ho *et al.*, 2012). A relatively new concept within is the development of even more complex co-culture spheroids, called 'organoids' which resemble miniature organs such as the kidney, liver and pancreas (Kermanizadeh *et al.*, 2014; Broutier *et al.*, 2016; Fatehullah, Tan and Barker, 2016; Henriksen-Lacey, Carregal-Romero and Liz-Marzán, 2016; Skardal, Shupe and Atala, 2016; Takasato *et al.*, 2016). Reconstructed tissues can exhibit characteristics that are very similar to native tissue in terms of morphology (*e.g.* fully stratified tissue), differentiation markers (*e.g.* ciliated cells, presence of tight junctions) and functional activities (*e.g.* mucus secretion, specialized cell for trans-epithelial transport, cilia beating, cell communications). These features are important in cellular uptake of NMs and cell behaviour in response to NM exposure. Reconstructed tissue models can therefore be used in conjunction with the aforementioned exposure configurations to inform on the transport of NM through biological barriers, such as the skin, placenta, intestine and lungs (des Rieux *et al.*, 2007; Vankoningsloo *et al.*, 2010; Hedwig M Braakhuis *et al.*, 2015; Lefebvre *et al.*, 2015; Kooter *et al.*, 2017; Vinardell *et al.*, 2017). Reconstructed skin models have received the most attention currently in the field of NM safety assessment, due to the widespread use of NMs in sunscreans and cosmetics, leading to the availability of a variety of 3D- reconstructed skin models, including but not limited to EpiSKIN and EpiDerm (Mathes, Ruffner and Graf-Hausner, 2014; Hayden *et al.*, 2015; Kim *et al.*, 2016; Wills *et al.*, 2016; Evans *et al.*, 2017; Hao *et al.*, 2017; Vinardell *et al.*, 2017). These models have been used in

conjunction with exposure to NMs such as MWCNTs and zinc oxides (ZnO) to determine the toxicity in these cell systems as opposed to dosing in standard 2D culture (Vankoningsloo *et al.*, 2010; Vinardell *et al.*, 2017).

Co-culture, organoid and reconstructed tissue approaches can be employed in microfluidic devices, such as the tissue and organ/organoid-on-chip approaches, and these 3D *in vitro* methods have already proven useful for measuring the penetration and translocation of NMs (Huang *et al.*, 2012; Albanese *et al.*, 2013; Bryce *et al.*, 2013; Bhatia and Ingber, 2014; Endes *et al.*, 2014; Kang *et al.*, 2016; Zervantonakis and Arvanitis, 2016). Future improvements to these microfluidic devices to include other dynamic processes such as perfusion and blood filtration, such as in the liver and kidneys, could improve further on the translation between these devices and the *in vivo* behaviour of NMs following exposure (Rennert *et al.*, 2015; Wilmer *et al.*, 2016). Another advantage of using reconstructed tissues is the possibility to mimic more realistic exposure scenarios in terms of NM presentation and dosimetry; although it is likely that the modelling of these NM transport processes will be more difficult than in static cultures. Indeed, some reconstructed human tissue (*e.g.* respiratory tract or epidermis) are cultivated in the ALI cell culture systems previously mentioned, allowing direct aerosol exposure systems for respiratory studies for both dispersed and dry nano-powders, or commercial formulations for cutaneous studies, thereby avoiding physical and chemical interactions that can occur in culture media (Fierz *et al.*, 2011; Mülhopt *et al.*, 2016; Kooter *et al.*, 2017). The dose is usually expressed as mass deposition per culture surface (µg/cm$^2$) but there are no standard protocols so far for the NM concentration range to test. For chemical regulatory toxicity testing, the OECD guidelines for cutaneous irritation assessment (OECD TG 439) recommend to uniformly cover the reconstructed epidermis surface with the test chemical and controls with a minimum dose of 25 µL/cm$^2$ or 25 mg/cm$^2$. Therefore, the physico-chemical parameters that affect NM deposition and cellular uptake and transport may have to been reconsidered in ALI tissue cultures.

Another interesting *in vitro* model that could be exploited for nanosafety studies is the use of precision cut tissue slices (PCTS). PCTS have already been established for drug toxicity testing: they allow reduced use of animals while testing compounds but maintain the cell complexity and architecture of a real tissue (Graaf, Groothuis and Olinga, 2007; de Graaf *et*

*al.*, 2010). The use of PCTS for nanosafety studies has not been fully exploited yet: while PCTS could bring several advantages because of the use of real tissue, particular care should be taken to develop methods to add the NMs to the PCTS resembling the way they would reach that particular organ *in vivo (Hirn et al., 2014; Merz et al., 2017)*, including any evolution of adsorbed biomolecules that would be associated.

## Conclusions

This review summarised the modes of exposure reported in NM toxicity studies, and the need for standardization and complete reporting of exposure conditions and experimental metadata to allow cross-comparability between studies. In particular the critical definition of dose in cell culture assay and tissue culture has been discussed and emphasized. Experimental conditions were highlighted that affect the local dose received by cultured cells in terms of number of NMs per cell, highlighting important considerations when conducting NM studies and indicating the minimal information to be included within studies to enable correlation of cellular received dose with observed toxicological response. The current state of the art in experimental settings and reporting in published nanotoxicity studies is often insufficient to derive the local dose and hence leaves room for discrepancies in the interpretation of dose-response relations. Standardized protocols for defined exposure are desirable, such that a higher level of comparability is achieved.

Therefore, to summarise there is a host of different options in terms of exposure configurations for NM investigations. The choice of which presentation method to use will depend on some key parameters namely: the type and form of NM that is to be investigated, the type of system to be modelled (i.e., ALI for toxicity testing of inhaled particles), and the parameters that need to be controlled for, such as NM agglomeration or pre-defined (or directly measured) NM dose. Each exposure setup offers different advantages and disadvantages summarised in Figure 3 and each allow precise control over different outcomes, or encompass simplified or more complex exposure methods. Regardless of the choice of exposure model, the information between different exposures must be reliable and reported in such a way that allows comparisons between different experimental designs. Therefore, we suggest the minimal required information for each exposure configuration as

presented below and summarised in Table 2. Our overall recommendations, regardless of the exposure configuration are as follows (see also SI for the brief protocol):

1. **Dispersion information**
    a. Calibration of sonicator and reporting of the critical DSE (optimized for the material to achieve suspension of the smallest possible aggregates)
    b. Concentration of the original dispersion
    c. Characterizations (minimum of TEM and DLS or DCS pre and post incubation in culture medium)
    d. Potential agglomeration over time and the effective density of agglomerates
    e. Dissolution in media over experimental time-course
    f. Corona characterization of NM in both conditioned and unconditioned media. We recommend the protocol described in (Albanese *et al.*, 2014)
2. **Cell information**
    a. Seeding density
    b. Density at exposure
    c. Cell size (area)
3. **Vessel information**
    a. Well plate format (6, 12, 24, 96 etc) and exact area of plate
    b. Shape of container bottom (round, flat etc)
    c. Volume and height of media used per well
    d. Material of container and binding potential
4. **NM exposure dose**
    a. Time length of incubation

b. Number and frequency of doses (single or multiple)

c. Total administered dose

d. Calculated local dose (particle number per area) and surface area of NM, based upon existing models that incorporate estimations of diffusion, sedimentation, dissolution and agglomeration if applicable (upright and submerged cultures).

These recommendations are based on the previously discussed issues surrounding difficulties in obtaining accurate comparisons between performed studies. By documenting all of the available and appropriate metadata, even if the local dose reported between papers is in a slightly different form, the availability of all the other exposure parameters allows readers to recalculate various aspects of the experimental set up and dynamics, in addition to improving consistency between experiments and therefore increasing result quality and comparability. The work performed within the QualityNano infrastructure has allowed development of robust SOPs capturing these factors: coupled with appropriate training of the researcher performing the tests, the developed SOPs allowed generation of highly reproducible data on NM uptake and cytotoxicity in independent laboratories. The SOPs (characterisation by DLS and DCS, quantification of cellular uptake of NMs by flow cytometry and cytotoxicity determined by the MTS assay, and the interlaboratory comparisons undertaken on them) are described in detail in articles included in this special issue (Langevin et al. 2017; Salvati et al. 2017).

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| **Particle Size (nm)** | **Preparation** | **Characterization** | **Cell density** | | **Exp Timing (h)** | **NM concentration** | **Vol of exposure media** | **Area** ($cm^2$) | **NM Cellular Dose** | **Ref** |
|---|---|---|---|---|---|---|---|---|---|---|
| 3-104 | Probe sonicated (750w, 20kHz, 29%) 10-15 s (x2) | DLS, TEM, BET | 100000 until 70-80% confluency | | 24 | 2-100 µg/mL | Not stated | 9.6/3.9/1.9 | Not known | Han 2012 |
| 15 | Probe sonicated 3 min. Stored at -20°C/ Son. 3x20 s (60w) | DLS, zeta potential | 10000 cell/$cm^2$ | 72 h | 24 | 5-160 µg/$cm^2$ | Not stated | 3.9 | Not known | Hussain 2009 |
| 20 | Milling | DLS, TEM, (XRD, BET previously) | 16000 cell/$cm^2$ | ~15 h | 24 | 150-3000 µg/mL | Not stated | 9.6/0.32 | Not known | Tedja 2012 |
| 5 | Sonicated 5 min | TEM, XRD, PCS, ICP-AES | 3000 cell/well | | 24/48 | 3-600 µg/mL | 200 µL | 0.32 | Could be calculated | Jin 2008 |
| 400 (measured by DLS) | Probe 10 min (10 x 1 min) 60w with ice water bath | DLS, Zeta potential, PDI, TEM | 10000-600000 cell/well | | 24 | 5-800 µg/mL<br>1-160 µg/$cm^2$ | Not stated | 25/0.32 | Not known | Hanot Roy, 2016 |

**Table 1.** Summary of the experimental information gathered from 5 research articles aimed at assessing $TiO_2$ uptake in cell lines. Taken from Han 2012, Hussain 2009, Tedja 2012 and Jin 2008, Hanot-Roy, 2016.

| | Preparation/ dispersion | Submerged cell culture | Inverted cell culture | Air liquid presentation | Surface presentation |
|---|---|---|---|---|---|
| **Parameter obtained in method** | Distributed sonication energy | Delivery by sedimentation and diffusion | Buoyant NM exposure | Delivered dose determination using QCM<br>Dynamic delivery<br>Bypasses agglomeration | NM dose known<br>Bypasses agglomeration |
| **To be reported** | Characterization (size, charge)<br>Agglomeration potential (modelled, estimated or measured) | Calculation of dose delivered (using model e.g. DeLoid 2017)<br>Necessary information to calculate dose (media height / total volume / well plate used, area)<br>Duration of exposure<br>Number of cells at plating and exposure / size of cells<br>Make and type of culturewear | | Report the recorded dose (QCM)<br>Cell number at plating and exposure<br>Information on culturewear (area of exposure) and conditions (make of transwell insert, membrane type)<br>Exposure duration | Report dose fabricated onto well<br>Cell number at plating and exposure<br>Exposure duration<br>Well type (make and size / area) |

**Table 2. S**ummary of how preparation and different NM exposure systems offer specific parameters and the different minimal information that needs to be reported in each case to ensure comparability of data generated.

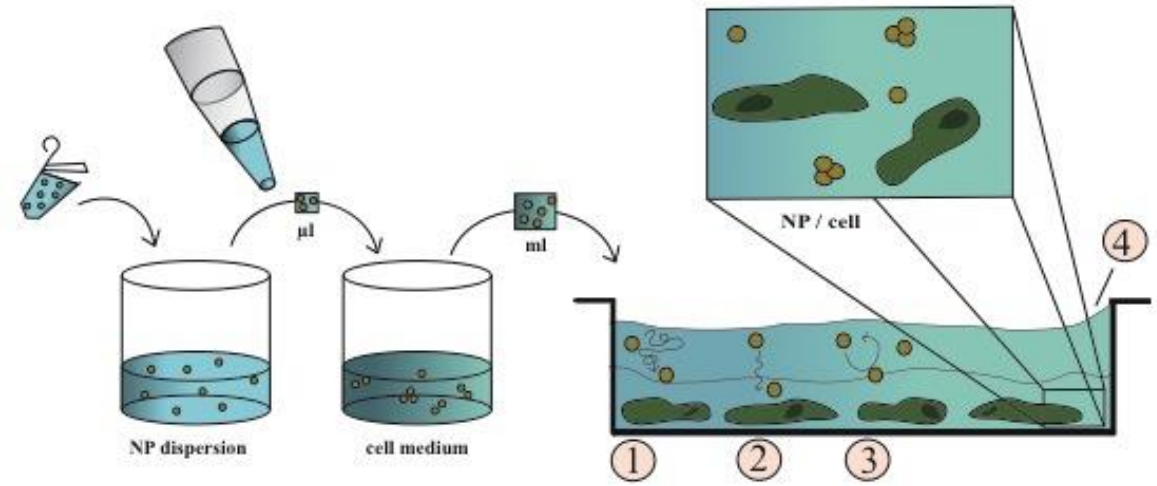


**Figure 1. The dosing of cells with NMs in an upright (standard) configuration.** The NMs must first be appropriately dispersed, diluted in the cell medium and then applied to cells. A variety of factors will impact on the transport rate of NMs to cells and therefore the delivered dose, including: 1) diffusion, 2) Sedimentation, 3) dissolution in media and 4) agglomeration state (both homoagglomeration with other particles and potentially heteroagglomeration with the walls of the exposure chamber).

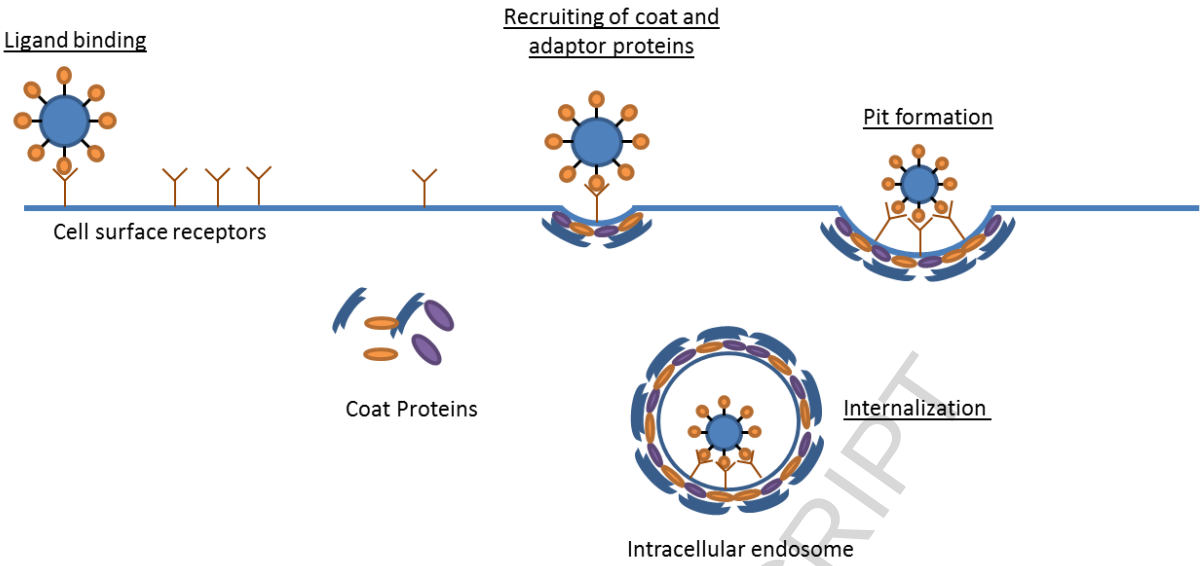


**Figure 2. Depiction of receptor mediated endocytosis** (adapted from (Banerjee, Berzhkovskii and Nossal, 2014). NMs coated in biomolecules come into contact with cell surface receptors of which there are a finite number of on the cell surface. Interactions between an NM surface protein and a receptor at the cell surface result in the formation of a NM-receptor complex. This complex can then recruit more cargo, coat proteins and adaptor molecules, increasing in size. These are then 'pinched off' to form intracellular vesicles that can transport within the cell through the endolysosomal system, where these NMs remain unless they manage to illicit endolysosomal escape.

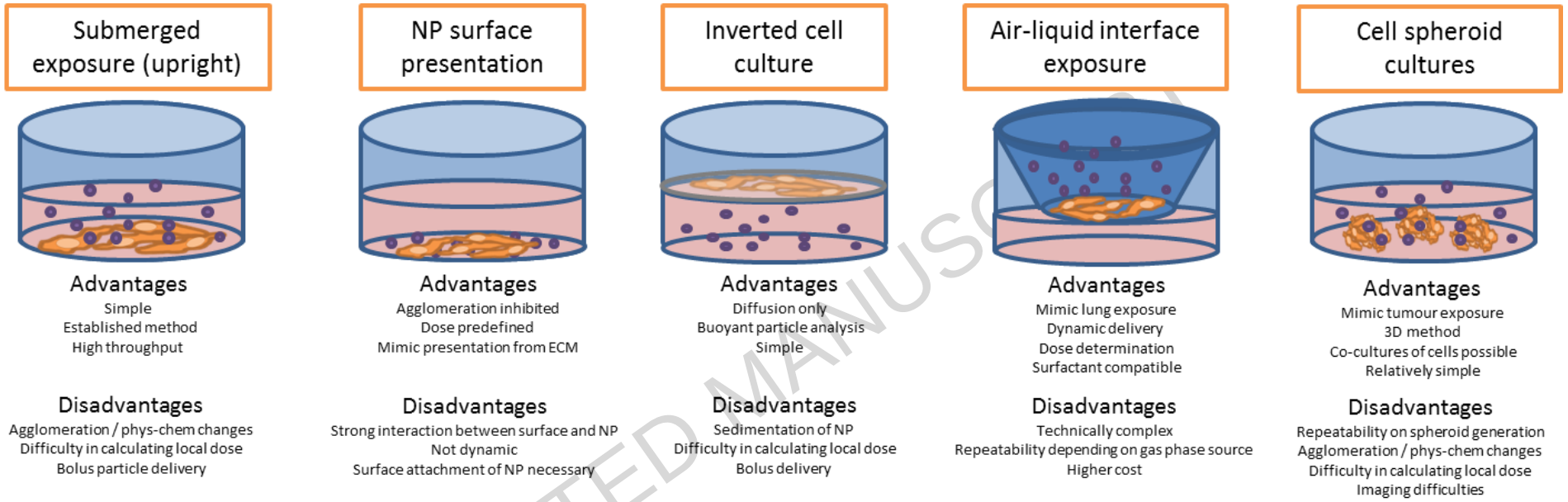


**Figure 3. Comparison of the available methods for exposure of NM in adherent cell cultures.** Methods include the described standard upright exposure configurations, NM surface presentation whereby NMs are fixed at the well surface and cells plated above the NM layer of known density. The inverted culture system is ideal for buoyant NMs, and the ALI system allows modelling of airborne exposure via specialised chambers. 3D spheroid/organoid generation is also depicted, which allows for mimicking of 3D culture systems resembling tumour delivery or complex organ structure, respectively. Some of the advantages and disadvantages or each approach are stated.

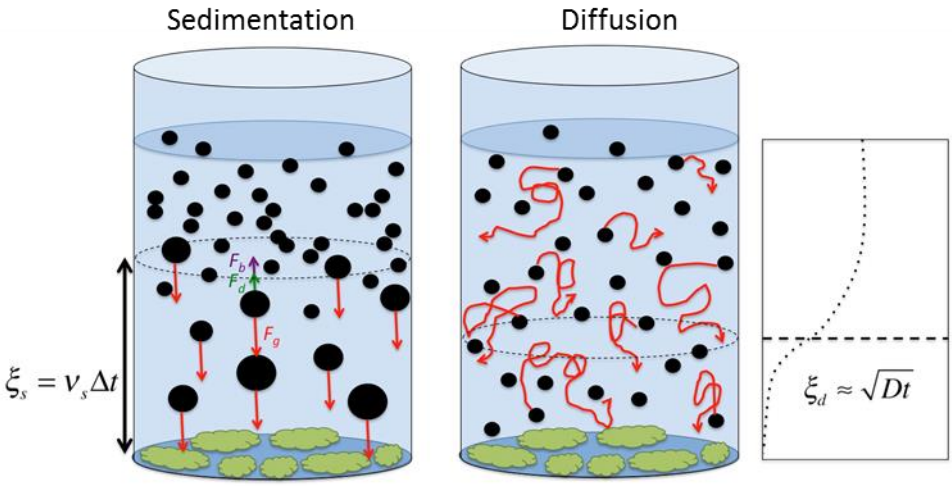


**Figure 4. Depiction of the sedimentation and diffusion of NMs.** Sedimentation varies with NM diameter and density and media density (drag force $F_d$ and buoyance $F_b$ opposite gravitational force $F_g$).The covered distance $\xi_s$ is determined by the sedimentation velocity $v_s$ and the time $\Delta t$. Diffusion depends upon the height of the media and the NM properties such as density and size. The covered distance $\xi_d$ is proportional to the square root of Diffusion $D$ and time $t$.

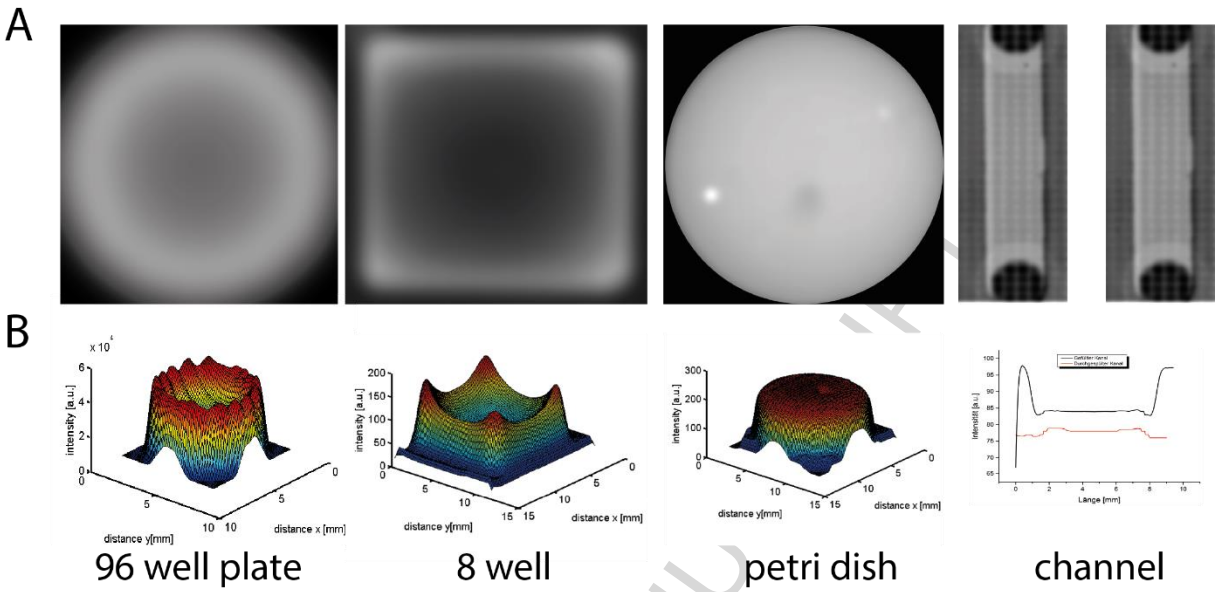


**Figure 5. Comparison of sedimentation of NMs in different submerged culture formats**

A) Fluorescent overview images of fluorescent PS-COOH NMs for 96 well plates, 8 well slides, petri-dish and channel slides (left: not rinsed right: after rinsing). B) Model of experimental data of rate of sedimentation of fibres as a function of the shape of the respective container. Open container (96 well plate, 8 well, petri dish) show meniscus effects and hence a inhomogeneous NM surface distribution, whereas closed containers (channel) exhibit a homogenous NM distribution.

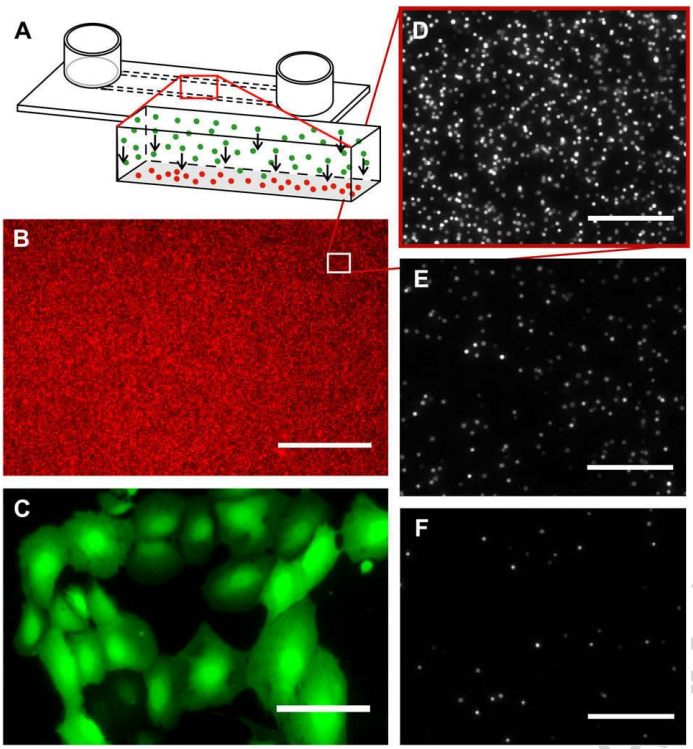


**Figure 6. Schematic representation of the NM surface preparation:** A. NMs are injected and sedimented inside a µ-channel in order to avoid meniscus effects. B, C. Homogeneous distributions of NMs (B) and cells (C) are achieved all along the channel. Scale bars correspond to 50 µm. D, E, F. NM surface density with varying NM concentration in solution (D) 100 pM, (E) 25 pM and (F) 12.5 pM. Scale bars correspond to 5 µm. (Reprint from (Alberola and Rädler, 2009)).

**REVIEW ARTICLE: Refining in vitro models for Nanomaterial exposure to cells and tissues**

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## Highlights

- Nanotoxicity assessment requires a definition of dose and standardized modes of exposure for nanomaterial uptake and cytotoxicity studies.
- Nanotoxicity studies often report only the exposure dose, which can lead to large variations in the *intrinsic* or *delivered dose* due to inhomogeneous exposure and loss of treatment material depending on how NMs are "*presented*" to cells.
- Protocols for NMM dispersion and cellular assay design therefore require standard operation procedures (SOPs).
- Experimental conditions including handling of nanomaterial (NM) dispersions as well as the order, timing and exposure configurations of the incubation can have an immediate effect on the resulting physical distribution of NMs on cell surfaces.
- Efforts to improve *in vitro* models such that they more closely mimic the *in vivo* conditions, include the Air-liquid interface approach, microfluidics and 3D cultures such as spheroids and organoids.