2 Methodological Considerations for Setting Up Human-Relevant In Vitro Nanotoxicology Experiments—A Practical Guide

Vânia Vilas-Boas, Emma Arnesdotter, Félix Carvalho, and Ernesto Alfaro-Moreno

Abstract

Nanotoxicology is a rapidly evolving field dedicated to assessing the safety and potential hazards of nanomaterials on human health. This practical guide outlines essential methodological considerations for designing human-relevant in vitro nanotoxicology experiments. A primary focus is placed on the comprehensive characterization of the nanomaterial in question, as properties such as size, shape, surface charge, and solubility significantly influence biological activity. The guide discusses the selection of appropriate in vitro models, including various cell sources, to ensure relevance to human exposure scenarios.

It is crucial to exercise caution when choosing test methods to account for potential nanoparticle interference with the selected assays; however, the use of suitable controls can help mitigate the impact of these interactions. The guide also emphasizes accurate practices for nanomaterial sample preparation and the importance of dosimetry, facilitating the translation of in vitro findings to realistic human exposure conditions. Guidance on exposure concentrations is provided to ensure that testing remains biologically and environmentally relevant. Furthermore, the guide includes reflections and perspectives on addressing

V. Vilas-Boas (✉) · E. Alfaro-Moreno
International Iberian Nanotechnology Laboratory, Nanosafety Research Group, Braga, Portugal
e-mail: vania.vilasboas@inl.int

E. Arnesdotter
Environmental Sustainability Assessment and Circularity (SUSTAIN) Unit, Luxembourg Institute of Science and Technology, Esch-sur-Alzette, Luxembourg

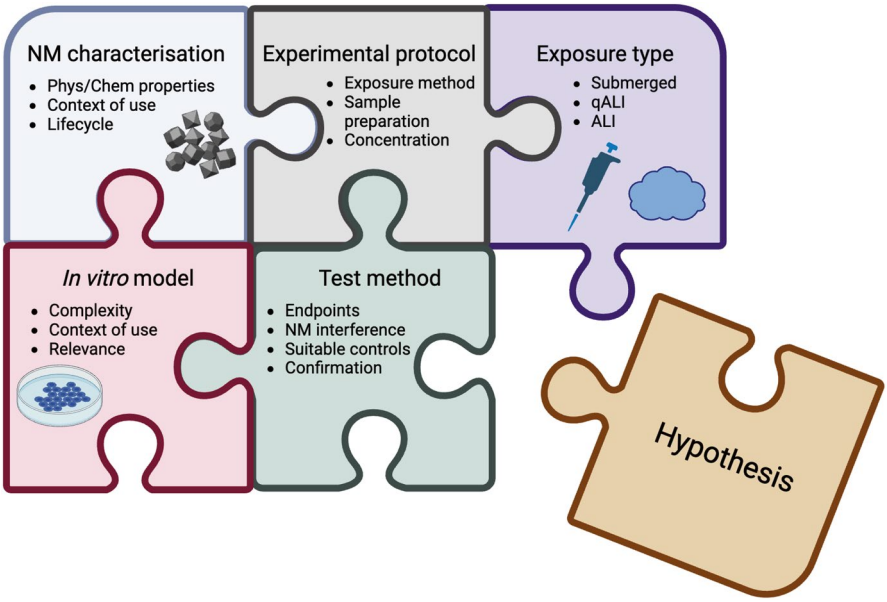
F. Carvalho
UCIBIO—Applied Molecular Biosciences Unit, Laboratory of Toxicology, Department of Biological Sciences, Faculty of Pharmacy, University of Porto, Porto, Portugal

Institute for Health and Bioeconomy (i4HB), Laboratory of Toxicology, Faculty of Pharmacy, University of Porto, Porto, Portugal

E. Alfaro-Moreno, F. Murphy (eds.), *Nanosafety*,
https://doi.org/10.1007/978-3-031-93871-9_2

common challenges and enhancing reproducibility in nanotoxicology studies. By adhering to these guidelines, researchers can generate more reliable and human-relevant in vitro nanotoxicology data, thereby supporting the risk assessment of nanomaterials.

Graphical Abstract



Keywords

Nanotoxicology · Advanced in vitro models · Nanomaterials · Test method · Experimental protocol

Abbreviations

ADME	Absorption, distribution, metabolism, excretion
ALI	Air-liquid interface
AO	Adverse outcome
AOP	Adverse outcome pathway
ELISA	Enzyme-linked immunosorbent assay
FBS	Fetal bovine serum
FDA	Food and Drug Administration
GIVIMP	Good in vitro method practices

IATA	Integrated approaches to testing and assessment
iPSC	Induced pluripotent stem cells
KE	Key event
LAL	Limulus amebocyte lysate
LBP	Lipopolysaccharide-binding protein
LDH	Lactate dehydrogenase
LPS	Lipopolysaccharide
MIE	Molecular initiating event
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NM	Nanomaterial
NP	Nanoparticle
OECD	Organization for Economic Co-operation and Development
OoC	Organ-on-chip
qALI	*Quasi* air-liquid interface
ROS	Reactive oxygen species
SOP	Standard operating procedures
TNFα	Tumor necrosis factor alpha
TRL	Toll-like receptor

1 Introduction

Since the emergence of nanotechnology in the1980's, a growing number of nanomaterials (NMs) has been discovered and developed for use in different sectors, including healthcare, agriculture, environmental, and energy applications (Xuan et al. 2023). An increasing number of consumer products, such as electronic devices, cosmetics, textiles, paints, and even food items, now incorporate NMs. This widespread usage raises the rate of human exposure, both directly and indirectly, through environmental accumulation. As exposure to potentially hazardous NMs rises, so does the associated risk. Therefore, it is crucial to evaluate the safety of NMs before their incorporation into consumer products or significant environmental accumulation, to prevent unwarranted public concern. While we possess some understanding of the general adverse effects associated with various types of NMs, our grasp of the underlying molecular mechanisms remains limited (Malakar et al. 2021). In recent years, adverse outcome pathways (AOPs)—schematic representations of the sequence of key events (KEs) from a molecular initiating event (MIE) to a measurable adverse outcome (AO)—have emerged as valuable tools for designing toxicity testing strategies in (nano)toxicology. These frameworks support the development of new testing methods and guidelines, as well as integrated approaches to testing and assessment (IATAs) (Gerloff et al. 2017). However, it is important to recognize that NM interactions with cells (or cellular targets, such as membrane receptors) may involve specific mechanisms, such as mechanical damage, which cannot be categorized as purely "molecular" interactions. This complexity may complicate the identification of a "molecular" initiating event, making it more appropriate to designate an initial KE that describes non-specific interactions. Nonetheless, the

progress made in this area demonstrates the feasibility of using AOPs to articulate the mechanistic knowledge of the toxicological pathways induced by poorly soluble NMs (Gerloff et al. 2017). Consequently, further research should focus on advancing nano-related AOPs and their application to predict the AO of new materials.

There has been a paradigm shift in toxicology in recent years, driven by increasing ethical constraints and acknowledged species differences between laboratory animals and humans. Studies indicate that data derived from rodents, the most commonly used animal models in preclinical toxicology, can predict or replicate less than 50% of human clinical toxicological outcomes for 150 compounds (Olson et al. 2000). While conventional in vitro models (2D monolayers) have significantly contributed to our understanding of nanotoxicology, they are not suited to address all research questions due to their simplistic and inaccurate representation of human physiology.

In recent years, remarkable advancements have been made in the development of models that more closely replicate the in vivo human situation when compared to traditional 2D monolayer cultures. These advanced models mitigate some limitations of 2D monolayers by employing strategies such as co-culturing different cell types on transwell inserts with permeable membranes to allow aerosol exposure (Klein et al. 2013; Ramos-Godínez et al. 2013) or assembling cells into spherical 3D structures known as spheroids (Bell et al. 2016; Vilas-Boas et al. 2021). Others have designed chip-like structures, termed organs-on-chip (OoCs), where cells are seeded and the culture medium is perfused using microfluidics (Huh et al. 2007), or mechanical cues are induced to simulate physiological movements (Stucki et al. 2018). OoCs mimicking the lung, liver, or heart have already been successfully applied in nanotoxicology (Lu and Radisic 2021). Additionally, organoids—3D heterogenous structures that grow from pluripotent stem cells, recapitulating real organs by recreating various cell types within an organ system—show significant potential to replace animal studies in the future (Clevers 2016). To leverage the strengths of both OoCs and organoids, researchers have recently combined these technologies to create 3D tissue replacements that self-organize from stem cells in a dynamically controlled environment, continuously monitored by integrated sensors (Zhao et al. 2024). The different complexities of these models allow researchers to address varied research questions, each with its own advantages and limitations, and not all complex models will be suitable for every study.

Nanotoxicology primarily applies principles of chemical toxicology to NMs. However, due to their small size, NMs possess increased surface area and reactivity, which not only contribute to NMs-related toxicity but also interfere with many cytotoxicity assays used for conventional chemicals. This interference presents a significant challenge in nanotoxicology, emphasizing the need for researchers to consider this aspect when designing experiments. Methodological choices should rely on preliminary tests to exclude NM interference with reagents and readouts (Karlsson et al. 2015).

To date, numerous studies have demonstrated that NMs can induce oxidative stress, leading to inflammation and cell death (Vilas-Boas and Vinken 2021). The emergence of new methods, such as diverse omics techniques, has facilitated the

identification of novel AOs triggered by NMs, as well as the underlying mechanisms involved.

Once NMs, in vitro models, and methodological approaches are selected, it is crucial to meticulously design the experimental procedure to address the research question and objectives while considering the challenges, possibilities, and limitations of the chosen methods. Careful design is essential, as inadequate experimental planning may lead to erroneous conclusions. Considerations must include the type of exposure, NM concentrations, the selection of appropriate positive and negative controls, and the necessity of confirmation assays.

In this chapter, we will discuss the major NM- and in vitro model-related aspects and key elements to consider when setting up an in vitro nanotoxicology study. Additionally, we will present the main methodological challenges associated with designing a robust and reliable nanotoxicological assessment.

2 The NM

2.1 Detailed Physical-Chemical Characterization of the NM

Factors such as size, surface area, charge, shape, and composition are crucial in determining the behavior and toxicity of nanomaterials (NMs). These characteristics often correlate with the cytotoxicity observed for specific NMs (Awashra and Młynarz 2023; Gerloff et al. 2017). For instance, positively charged NMs (i.e., those with a positive zeta potential) are generally more likely to interact with negatively charged cell membranes compared to negatively charged or neutral NMs. Additionally, different adverse outcomes or severities may be expected from NMs with average primary particle sizes of 5 nm versus 100 nm (Gerloff et al. 2017). Smaller NMs are typically more readily taken up by cells, which may result in more pronounced toxicity. Likewise, if a NM is partially soluble, toxicity related to released ions should also be considered (Drasler et al. 2017). A recent review by Ruijter et al. (2023) summarizes how NM characteristics influence their toxicity in in vitro studies.

While there is now a greater awareness of the importance of understanding the characteristics and behavior of NMs, the lack of such knowledge has historically been identified as a significant drawback. Even today, most available physicochemical data pertain to pristine NMs and do not account for changes that cell culture media (or other exposure vehicles) may induce in the inherent properties of the NMs. Moreover, the expected biotransformation of NMs in their environment before reaching their target—such as the transformations occurring in the gastrointestinal tract upon ingestion—will likely impact their kinetics in the body, including absorption, distribution, metabolism, and excretion (ADME) (Gerloff et al. 2017). Therefore, these factors must be considered when designing experiments intended to reflect real-life scenarios.

While data on particle composition, size, and shape can be obtained from pristine materials, it is essential to characterize the NM when dispersed in the exposure

medium, as this is how it will encounter cells or tissues (Drasler et al. 2017). In this context, information on hydrodynamic size, solubility, aggregation, and the presence of contaminants, such as endotoxins, becomes equally essential (Swartzwelter et al. 2021). It is also vital to note the distinction between aggregates—particles comprising strongly bonded or fused entities—and agglomerates, which are collections of weakly bound particles, aggregates, or mixtures of the two (OECD 2012). For detailed information on the main techniques used for NM characterization, refer to Drasler et al. (2017).

2.2 Endotoxin: A Biological Component that Must Be Kept in Mind

Endotoxin is a molecule found on the surface of many materials; it is a heat-stable, pyrogenic complex component of the membrane of Gram-negative bacteria (Raetz and Whitfield 2002). Specifically, endotoxin is a lipopolysaccharide (LPS) heteropolymer composed of three elements: lipid A, core oligosaccharide, and O-specific polysaccharide, also known as antigen-O (Erridge et al. 2002). While LPS is a component of endotoxin, it is common for these terms to be used interchangeably, even though they are not synonymous. The heat-stable nature of endotoxin makes it difficult to eliminate from a wide range of materials (e.g., autoclaving does not effectively remove endotoxin). Thus, it is essential to ascertain the presence or absence of endotoxin when evaluating the toxicity of NMs.

Endotoxin binds to various cellular receptors, including toll-like receptor 4 (TLR4), LPS-binding protein (LBP), and CD14 (Ulevitch and Tobias 1995). The interaction of these receptors with endotoxin triggers a cascade of cellular events that leads to the release of proinflammatory mediators such as TNFα, IL-1β, IL-6, prostaglandins, and leukotrienes (Beutler and Rietschel 2003). For decades, evaluating endotoxin presence has been a standard practice when assessing respirable particles (Griwatz and Seemayer 1995), and its role in complex mixtures has been clarified using specific inhibitors such as LBP (Bonner et al. 1998) or Polymyxin B (Alfaro-Moreno et al. 2007). In the context of assessing NM toxicity, several studies have underscored the importance of evaluating endotoxin presence. For example, Dobrovolskaia et al. (2010) demonstrated that the presence of endotoxin in carbon nanotubes could lead to an overestimation of the NMs' cytotoxicity (Dobrovolskaia et al. 2010). Similarly, a review by Deng et al. (2009) emphasized the necessity for standardized protocols and guidelines to assess endotoxin contamination in NMs, as inconsistent or inadequate evaluations can yield unreliable toxicity data (Deng et al. 2009).

To address these concerns, various methods have been developed for detecting and quantifying endotoxin in NMs. Historically, the Limulus Amebocyte Lysate (LAL) assay, which utilizes the clotting cascade of the horseshoe crab Limulus polyphemus, has been the most commonly used method for endotoxin detection and quantification (Colas et al. 2014). However, a comparable method produced without the need for animal-derived raw material is now available, called Recombinant

Factor C, which is based on the same reaction principle as the LAL assay (Bolden and Smith 2017). Other techniques, such as mass spectrometry and nuclear magnetic resonance spectroscopy, have also been explored for identifying and characterizing endotoxin in NMs (Bergstrand et al. 2006; Gorbet and Sefton 2005).

In addition to analytical methods, it is crucial to develop strategies for removing or minimizing endotoxin contamination in NMs. These strategies may include thermal treatment, chemical modifications, and affinity-based purification techniques (Bacher et al. 2001; Sharma et al. 2014); however, no standardized methods for endotoxin removal from NMs currently exist (Hannon and Prina-Mello 2021). Consequently, evaluating the presence of endotoxin in NMs is a critical aspect of toxicity assessment, as this immunogenic molecule can significantly influence the interpretation of experimental results. By implementing robust protocols for endotoxin detection and employing strategies to prevent contamination, researchers and regulatory agencies can enhance the reliability and accuracy of NM toxicity data. This approach ultimately contributes to the safe development and application of these emerging technologies. More details on the relevance and impact of endotoxin on nanosafety assessments can be found in Chap. 3.

2.3 The Context of Use and Life Cycle

Knowing the intended use of NMs helps anticipate possible exposure scenarios and routes, as well as the main target organs or tissues. The primary routes of exposure to NMs are ingestion, dermal contact, inhalation, and occasionally injection (Fig. 2.1). Different exposure routes impact various organs in distinct ways. For example, when inhaled, NMs enter the nasal cavity, travel through the upper airways, and eventually reach the lungs. The size of the inhaled particles determines their final destination, while their surface charge and functionalization may also affect absorption, emphasizing the relevance of detailed physicochemical characterization of the NM (Savage et al. 2019).

Some organs or tissues, such as blood and liver, are generally at risk of exposure due to their interaction with the exposure route. The liver often accumulates and transforms NMs (Li et al. 2022) making it a crucial to study cytotoxic effects in liver models. The brain, fetus, reproductive system, and endocrine system can also be exposed once NMs enter the bloodstream, increasing the importance of assessing the potential effects on these organs. There has been growing interest in studying the endocrine disruption ability of NMs, revealing that ubiquitous materials, such as diesel exhaust NPs, may disrupt hormonal regulation in both men and women, although the effects might stem from the chemical mixture they carry (Iavicoli et al. 2013).

If the NM is intended for health applications (e.g., as a drug carrier), it is advisable to consult the literature and market for qualified models that have undergone regulatory acceptance for toxicological assessment. This approach will increase confidence in the results and expedite the review process by regulatory agencies.

Sources of NMs	Cosmetics, textiles, cleaning prod, painting, hairdressers, agriculture, etc	Gases, vapors, mists, fumes, dusts, in environmental or occupational scenarios	Food additives, environment-contaminated food, contaminated water	Therapeutics, cosmetics
Exposure route	Dermal	Inhalation	Ingestion	Injection
Entrance point in the body	Skin/eyes	Airways (from nose to lungs, depending on size), brain (via olfactory bulb)	Digestive tract (from mouth to intestine, depending on size, charge, etc)	Muscle, fat, skin, blood
	Blood/vascular tissue			
Other target organs or systems	Lymphatic system, fat	Heart, liver, kidneys, brain	Mainly liver due to first passage, but then heart, lungs, kidneys, brain	Liver, heart, lungs, kidneys, brain
Suggested exposure method	1. ALI (aerosol or powder) 2. qALI 3. SUB	1. ALI (aerosol or powder) 2. qALI (e.g. in artificial lining fluid) 3. SUB	SUB	SUB

Fig. 2.1 Examples of NMs sources, their exposure routes, and main target organs implicated in the exposure. Based on this information, an in vitro exposure scenario is suggested

3 The In Vitro Model

The choice of the in vitro model used for experiments is a critical component of the setup. A careless selection may result in significant project costs without justifiable outcomes. For a thorough toxicity assessment, the selected in vitro model must express the cellular targets involved in the suspected pathways or mechanisms of toxicity, regardless of the model's complexity. In this subsection, we will outline the key considerations for selecting an appropriate in vitro model for a predefined study.

3.1 The Context of Use

The context of use refers to the applicability domain and specific purpose of an in vitro model or method, as outlined by the FDA (FDA 2017). Understanding this context, along with a thorough knowledge of the limitations, sensitivity, reproducibility, and relevance of the models and assays, is essential for obtaining high-quality results, especially in a regulatory framework. These parameters should be clearly defined for both the in vitro model and the selected test method to build confidence in the results.

Exploring the wide range of available complex in vitro models may help identify one that meets your specific needs. Collaborating with model developers can enhance focus and optimize collective efforts. If you choose to develop a new model, consider conducting experiments not only centered on your primary research question but also specifically aimed at validating or qualifying the new model. This

approach will ensure its acceptance and adoption by end-users, such as pharmaceutical companies (FDA 2017).

To facilitate this process, it is crucial to adhere to the official guidelines set forth by the OECD for the validation of methods and models (OECD 2005). Note that the 2005 document is currently under revision and will be updated soon.

3.2 Cell Source

Various cell types can be employed to establish in vitro models, ranging from cell lines to primary cells and stem cells. The choice of cell type can significantly influence the outcomes related to the original research question, with some options being more relevant to the human context than others. Primary human cells are generally regarded as the gold standard, particularly in liver toxicology, as they retain characteristics of the original tissue depending on the model's configuration (Zhao 2023). When possible, primary human cells should be the preferred choice for obtaining human-relevant responses at a population level, as they can partially account for inter-donor variability (Vilas-Boas et al. 2021). However, primary cells are often expensive, have limited availability, and are subject to ethical restrictions. Additionally, they typically originate from patient biopsies, which carry inherent risks, especially since these procedures are usually performed under the suspicion of disease.

Recently, significant efforts have been made to develop stem cell-based models, such as organoids, using tissue-resident stem cells from patient biopsies or induced pluripotent stem cells (iPSCs). iPSCs are created by reprogramming adult somatic cells through the overexpression of the so-called Yamanaka factors (Lynch et al. 2019). This approach offers an abundant source of cells with differentiation potential, enabling the recreation of various cell types from the same initial source. However, these models have yet to fully replicate the maturity and cellular diversity found in most adult tissues (Liu et al. 2017). Therefore, further optimization of stem cell-based, human-relevant in vitro models is essential.

In many cases, immortalized cell lines are the most convenient option, providing an unlimited source of human-derived material and expressing key features that make them suitable for quickly addressing important mechanistic questions in a simplified setup. These immortalized cell lines can also be combined to enhance model complexity and more closely resemble the in vivo conditions of human tissues. It is crucial to note, however, that most available cell lines are derived from tumor tissues, which may not always be ideal, as metabolic pathways can be upregulated and cell communication pathways downregulated compared to primary tumor cells or normal cells (Ertel et al. 2006). Fortunately, an increasing number of cell lines derived from normal human tissues are becoming available, presenting a viable alternative.

It is important to recognize that both stem cells and immortalized cell lines do not account for inter-individual variability in human responses. While this limitation allows for more definitive observations regarding a specific genotype or

phenotype represented by a particular cell line, it fails to capture the inherent variability of the human population.

3.3 Model Complexity

The susceptibility of cells to NMs depends not only on the cell type but also on cell architecture and model complexity (Juarez-Moreno et al. 2022). For decades, in vitro toxicity studies primarily relied on simple 2D monolayers of a single cell type, typically of cancerous origin. While these models are overly simplistic and do not accurately reflect human physiological complexity, they have been instrumental for screening purposes and in enhancing our understanding of the metabolic pathways and molecular mechanisms triggered by chemicals (Faber and McCullough 2018; Gómez-Lechón et al. 2014). Consequently, simpler models may be more suitable for addressing specific molecular questions compared to more complex systems (Table 2.1).

Recently, there has been a great incentive to develop models that more closely resemble human physiology, such as OoCs and organoids, resulting in an abundance of new complex models for toxicological studies (Leung et al. 2022). These models often integrate multiple cell types and physiological cues, such as perfusion and stretch/strain, making them more appropriate for understanding toxicological effects related to interactions among cells, the extracellular matrix, and different organ systems. For example, co-cultures that include immune cells are generally better suited for studying inflammatory responses triggered by chemicals, including NMs. Similarly, an OoC combining two tissues in tandem may be valuable for assessing the indirect adverse effects on a secondary organ or tissue mediated by messenger molecules released by cells directly exposed to NMs. Additionally, ongoing efforts aim to integrate organoid and OoC technologies, allowing organoids to be exposed to physiologically relevant controlled conditions (Zhao et al. 2024).

It is important to note, however, that most of these more complex models currently exhibit lower throughput and reproducibility. This factor should be taken into account when selecting the appropriate in vitro model for specific studies.

Table 2.1 Association between complexity, type of exposure and context of use of in vitro models for nanotoxicology

Model complexity	Single or co-cultures				Self-generated
	2D	Polarized		3D	
	Monolayers	Inserts	(m)OoCs	Spheroids	Organoids
In vitro exposure	SUB	SUB, ALI, qALI		SUB	
Context of use	Molecular mechanisms	Barrier tox			
	Tox screening	(inter-)tissue-level tox			
	Cell-level tox		(inter-)organ-level tox		

ALI air-liquid interface, *m* multi, *OcO* organ-on-chip, *qALI* quasi-air-liquid-interface, *SUB* submerged, *Tox* toxicology

More information on advanced models for nanotoxicology assessments can be found in Chap. 7 of this book.

4 The Test Method

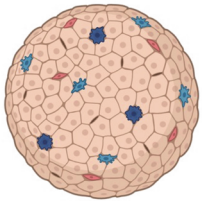
4.1 The Outcome

The endpoints to be assessed should align with the research question being addressed. In this context, available AOPs in nanotoxicology can be valuable tools for identifying individual KEs involved in the anticipated pathways. The preferred assay should also facilitate the quantitative detection of the perturbation (in this case, a KE) caused by the stressors (positive control and/or the NM under analysis) in the selected in vitro model. Additionally, the chosen test method should align with the context of use for which it was developed (Hirsch and Schildknecht 2019). Consideration should also be given to the ease or difficulty of sample collection from the selected in vitro model, and vice versa. Other criteria to account for include the assay's predictiveness, robustness, readiness, simplicity, and cost (Ruijter et al. 2023).

Oxidative stress, inflammatory response, cytotoxicity and apoptosis induction, as well as genotoxicity, are outcomes well documented to be triggered by various types of NMs and should be prioritized when testing new NMs (Drasler et al. 2017; Ruijter et al. 2023; Vilas-Boas and Vinken 2021). As discussed in Sect. 2.1. and Chap. 3, it is particularly important to check for endotoxin contamination when studying inflammatory responses, as its presence can trigger inflammation independently of the NM, complicating the ability to discriminate effects purely attributable to the NM.

Research can focus on either the bulk response related to the function of the whole cell population or tissue (e.g., cell viability, membrane integrity) or on the mechanisms behind those outcomes (molecular response). For more detailed studies, it may be necessary to knock-down the cellular expression of relevant proteins or to use inhibitors of specific pathways to elucidate the toxicological pathway triggered by the NM. Bulk responses consider the tissue as a unit, where the most prevalent cellular response is perceived as general. In this scenario, distinctive individual cell responses, which may be significant, are often overlooked. If this approach does not adequately address the initial research question, techniques such as flow cytometry or fluorescence microscopy can help distinguish responses from different cell types, though they are limited to a small number of proteins and mRNAs.

Ultimately, single-cell analysis techniques, such as single-cell omics (transcriptomics, genomics, metabolomics, proteomics), may be required to gain a deeper understanding of the role of each cell in a complex tissue model. Importantly, while bulk analysis methods are generally more affordable and easier to perform in standard biochemistry laboratory settings, single-cell techniques tend to be more costly



	Bulk response		Single-cell response
	Functional	Molecular	
Outcomes or techniques	•Cell viability/death •NP internalization •Cytokine release •ROS production	•Omics •Protein knock-down •Transporter inhibition	•Single-cell omics •Patch-clamp recording •Flow cytometry •Fluorescence microscopy
Advantages	•Tissue-level response		•Highlights cell-to-cell variation •More data from the same cell •Multi-omics available from the same cell •Lower sample amount
	•Speedy •High throughput •Affordable •Simpler data analysis	•Specific cellular targets •More sensitive to sublethal responses •Mechanistic clues	
Limitations	•Cellular heterogeneity is concealed	•Laborious •Intermediate cost •High variability of results	•Challenging sample prep •Onerous data analysis •Labour intensive •Higher cost

Fig. 2.2 Advantages and limitations of analyzing bulk adverse response comparatively to single-cell response in complex in vitro models

and complex to implement, yielding more intricate data, and requiring specific equipment not readily available in every lab (Fig. 2.2).

As a rule of thumb, starting with functional assays and subsequently integrating more single-cell-oriented assays will help determine the necessity for incorporating complex single-cell analysis in subsequent experiments.

4.2 Interference of the NM with Test Methods

It is crucial to exclude potential interferences of the NM with the test method readout to ensure reliable results. This step is fundamental for validating the experiment's outcomes. NMs have been known to interfere with several methods, including the MTT assay, which remains one of the most commonly used assays (Awashra and Młynarz 2023; Kroll et al. 2012; Lebre et al. 2022).

Interference of NPs with test methods can occur in several forms, including:

- Physical interference due to the light-refracting or light-absorbing properties of certain NMs, leading to increased absorbance or decreased fluorescence due to quenching phenomena. If the interference is purely physical, it can generally be addressed by adding a centrifugation step to separate the supernatant for reading, or by subtracting the background absorbance or fluorescence generated by the cells in the presence of the NM (Stone et al. 2009).

- Physical interference from the binding of biomarkers of interest present in the cell culture medium to the NM surface. The increased surface area of NMs provides more docking sites for proteins in the milieu. This is particularly relevant when investigating molecules of the secretome, which are released by cells into the extracellular medium. For instance, it has been documented that NPs can interfere with biomarker detection protocols, such as ELISA, as the cytokines released into the medium may bind nonspecifically to the NP surface (Guadagnini et al. 2015).
- Chemical interference when the NM interacts directly with the detection reagent. Due to their small size and increased surface area, NMs can exhibit heightened reactivity, which may impact assay results. For example, in methods based on cellular redox activity, such as the MTT assay or the generation of ROS, it is essential to assess the direct catalytic effects of NMs beforehand (Awashra and Młynarz 2023). To mitigate this interference, one option is to remove the NM remaining in suspension and wash the cells/tissue before adding the detection reagent. Alternatively, accounting for the signal obtained when mixing the NM with the reagent in the absence of cells may be helpful; however, it is often advisable to employ a different test method based on an alternative assay principle.

To identify potential interference of the NM on the selected assay, the test protocol should be conducted beforehand without cells to detect any reaction attributable solely to the NM. For instance, to assess interference with the method's readout or chemical reactivity with the reagent, various concentrations of the test NM can be mixed with the detection reagent in the absence of cells. These mixtures should be incubated for the expected assay duration, and the resulting assay signal (fluorescence, absorbance, or luminescence) should be measured (Vasimalai et al. 2018). If a signal change is observed compared to blank wells, it indicates that the reagent is affected independently of the presence of cells, confirming interference.

Since interferences are both assay- and NM-dependent, anticipating them without testing is nearly impossible (Karlsson et al. 2015). Therefore, it is highly advisable to perform exploratory tests to exclude such interferences in advance. Table 2.2 lists reported interferences of NMs with some of the most commonly used cytotoxicity assays, their causes, and possible solutions. Guadagnini et al. have reviewed this topic, highlighting other types of NM interferences for different assays (Guadagnini et al. 2015).

4.3 Control Samples and Assay Conditions

A good planning of the assay controls is crucial for obtaining reliable and robust results. Therefore, it is essential to define both positive and negative controls for each adverse outcome or assay. Ideally, these controls should be particulate to match the characteristics of the NM. However, finding nano-specific reference controls remains a challenge, as they must be specific to the endpoint under evaluation and compatible with the selected assay and exposure route (Drasler et al. 2017). In the

Table 2.2 Examples of interferences from nanomaterials with widely used toxicological assays

	Observed interference		
Methodology	Cause	Result interpretation	Proposed solution
MTT reduction WST reduction	NM optical density NM aggregation in cell medium	Falsely ↑ viability	Centrifugation step after cell lysis
	NM direct redox activity	Falsely ↑ **or** ↓ viability	Choose test not based on redox activity
LDH leakage	NM catalyzes the reaction in the absence of LDH	Falsely ↓ viability	Choose different test
ELISA (cytokine release)	NM adsorb protein of interest	Falsely ↓ cytokine production	Add serum proteins to NM suspension
Comet assay	NM interfere with enzyme activity NM induced breaks in naked DNA	Falsely ↓ genotoxicity Falsely ↑ genotoxicity	Choose different test
ROS quantification (H_2 DCF-DA)	NM direct redox activity	Falsely ↑ **or** ↓ ROS levels	Choose different test
	NMs quench fluorescence NMs scatter emitted fluorescence	Falsely ↓ ROS levels	Sample centrifugation after cell lysis

Adapted from Lebre et al. (2022)
NM nanomaterial, *MTT* 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, *LDH* lactate dehydrogenase, *WST* water-soluble tetrazolium salts, *ELISA* enzyme-linked immunosorbent assay, *ROS* reactive oxygen species, *H2DCF-DA* 2′,7′-dichlorodihydrofluorescein diacetate

absence of a nano reference control, bulk material can serve as a negative control (Wiemann et al. 2016), and at least one conventional chemical should be used as a quality control. Additionally, a non-exposure ("non-treated") control is imperative, where cells are added the same vehicle/dispersant and subjected to the same conditions as those in which the NM is exposed (Drasler et al. 2017). If different time points are being assessed, non-exposure control wells should be included for each time point.

4.4 Confirmation Assay

Given the numerous variables involved in assessing the toxicological profile of a NM in vitro, it is advisable to include a confirmation assay to validate findings. This is particularly crucial if the potential interference of the NM with the assay was not assessed beforehand. A confirmation assay may be based on the same mechanistic principle but, preferably, using a different readout. For instance, metabolic activity assays that measure different metabolic products can be employed. These assays are available with both optical and fluorescent readouts. In metabolic activity testing, such as the MTT reduction assay and resazurin reduction-based assays (e.g., PrestoBlue) decreased signals (absorbance for MTT and fluorescence for resazurin)

indicate decreased metabolic activity, suggesting decreased cell viability. An interference with the tests' readouts is expected to affect both absorbance and fluorescence in opposing ways, leading to conflicting cell viability results and raising concerns that factors other than toxicological effects may be influencing the outcomes. Ideally, confirmation tests should be based on different mechanisms (e.g., metabolic function and membrane permeability), and different readouts, to exclude interferences with the mechanistic pathway under analysis.

5 The Experimental Protocol

5.1 Exposure Method

There are various ways to present NMs to cells. Many in vitro experiments have traditionally utilized submerged (SUB) exposure, where cells are fully immersed in the medium containing the test NM. For NMs exposed in SUB conditions, the effective (actual exposure) concentration does not necessarily equal their available (nominal or applied) concentration (see subsection on dosimetry below). Advances in technology and cell model development now allow for exposure at the air-liquid interface (ALI), where the cell layer is in direct contact with air and not covered by cell culture medium. This approach more closely mimics real-life exposure scenarios of organ systems in direct contact with air, such as the airways, skin, or cornea, compared to SUB exposures.

Specific exposure systems, such as the VITROCELL Single Droplet Systems, enable direct exposure of cells at the ALI to various airborne substances, including NMs, brought into suspension. Platforms like the PreciseInhale, developed by Inhalation Sciences, or the PowderX from VITROCELL, allow for exposure to dry aerosols directly applied to the cells under high pressure. These systems eliminate the need to prepare a suspension of the NM before exposure, which can be beneficial for certain NMs that are susceptible to tangling and agglomeration. However, these exposure systems can be costly and may not be readily available in all labs.

As a compromise, the quasi-ALI (qALI) approach, sometimes referred to as pseudo-ALI, can be employed. In this case, the in vitro model is airlifted as in a regular ALI scenario, but the NM is delivered on the cells using a strictly limited amount of medium just to cover the surface (e.g., 50 $\mu L/cm^2$). Similarly, the Tecan D300e digital dispenser, primarily developed for chemicals, can be used to deliver NPs <1 μm at a concentration of ≤0.5% using as little as 1.3 $\mu L/cm^2$.

It remains challenging to determine the effective exposure concentration when using the systems discussed, as individual materials behave differently. This highlights the importance of a careful characterization and consideration of the test material. Nevertheless, with proper reporting and execution of experiments, all methods discussed should provide reproducible and accurate comparisons between NM studies in vitro.

5.2 Sample Preparation

Defining a stepwise process for preparing NM dispersions is crucial for obtaining reproducible results that lead to robust conclusions. Once the exposure method is selected, several considerations must be made regarding sample preparation to strike a balance between technical requirements and the relevance of the exposure for extrapolating real-life conclusions. After defining the exposure method, carefully consider the following sample preparation steps:

- **Use of Fetal Bovine Serum (FBS) as a Medium Supplement**: FBS provides essential nutrients for cell culture and may also act as a stabilizer/dispersant for some NMs by forming a corona of serum biomolecules (Casals et al. 2010). This protein corona can influence cell-NP interactions, with debates surrounding whether it enhances or decreases NM cytotoxicity (Corbo et al. 2016). On one hand, this biological coating may improve the biocompatibility of the material by blocking surface reactivity or decreasing the NM's surface energy (Vranic et al. 2017). Different amounts of FBS can lead to variations in the protein corona and may trigger distinct uptake mechanisms (Francia et al. 2019). If mimicking blood is the goal, using human plasma instead of serum might be more appropriate, as silica NPs, for example, can adsorb various coagulation factors at their surface (Aliyandi et al. 2021). Conversely, an increased biological component may enhance uptake, potentially raising cytotoxicity, particularly in immune system cells (Corbo et al. 2016). While short exposure periods may be feasible without FBS, it may be necessary for maintaining certain in vitro models in longer-term experiments. Thus, the decision to include FBS should be guided by the characterization of the NPs, the selected exposure method, and the specific needs of the chosen in vitro model. Appropriate controls should be included to rule out any detrimental effects on the biological system arising from the absence of FBS.

  In specific cases, such as respiratory airway exposure, the presence of serum might distance the exposure conditions from real-life scenarios, where NMs would not have a serum-derived corona at the time of exposure (Hsiao and Huang 2013). Consequently, including FBS could lead to an underestimation of some NMs' cytotoxic potential, despite contradictory findings in studies examining NM toxicity in the presence or absence of FBS (Hsiao and Huang 2013; Murugadoss et al. 2020; Vranic et al. 2017). In such cases, a simulated respiratory tract lining fluid, supplemented or not with lung surfactant, may serve as a more suitable dispersion medium (Kumar et al. 2017).
- **Homogenization Techniques**: Some NMs require specific dispersion steps to achieve a homogeneous suspension while balancing the preservation of the NM's properties with the homogeneity and stability of the dispersion. Dispersion steps will vary based on the inherent characteristics of the NMs. For instance, hydrophobic NMs require a pre-wetting step—creating a paste with ultrapure water or a small percentage of ethanol—before sonication to facilitate dispersion in aqueous medium (Hartmann et al. 2015). Direct sonication of NMs in cell culture

medium is strongly discouraged, as it may denature proteins and generate ROS from sonolysis. Recently, a milder dispersion protocol for hydrophobic NMs has been described, involving continuous stirring of the NMs in cell culture medium with FBS or bovine serum albumin (Lizonova et al. 2024). In this case, the stability of the NM dispersion is supported by the formation of a protective protein corona. A clearly defined sample preparation protocol will yield reproducible NM suspensions, increasing the reproducibility of results from repeated experiments (Ruijter et al. 2023). Several European projects, such as Nanogenotox and Nanoreg, have tackled this issue and generated dispersion protocols based on sonication, which are freely available for use. Note that acoustic energy and de-agglomeration effects can vary between different sonicator brands and even among the same models. Small procedural differences—such as operator technique, water quality, and temperature—can also influence results. However, sonication might not be suitable when attempting to mimic inhalation conditions or for NMs whose physico-chemical properties (agglomeration, dissolution, etc.) change during the process, as this may impact their toxicity. Other dispersion methods proposed in the literature include the use of detergents or surfactants (e.g., Tween-80, Pluronic), but these have been associated with adverse effects, including potential mutagenic activity (Drasler et al. 2017). Particle suspensions should be stable for at least 30–60 min post-preparation and should be freshly prepared for each experimental repetition, as suggested in the Nanogenotox protocol (Jensen et al. 2009).

- **Sampling from Real-Life Scenarios/Products**: Whenever possible, NMs should be sampled from real-life scenarios or products to enhance the relevance of results related to occupational exposures. More information and practical tips on sampling NMs can be found in (Hyun Lee et al. 2010).
- **Mimicking Biological Processes**: Biological processes should be mimicked whenever feasible. For instance, when investigating the effects of NMs or measuring NM uptake in the intestinal system, it is essential to consider the biotransformations that occur during human digestion before NMs interact with intestinal cells. This includes changes in surface chemistry and biocorona formation. In vitro gastrointestinal digestion protocols, such as INFOGEST 2.0 (Brodkorb et al. 2019), replicate protein digestion using standard laboratory equipment. In this process, materials—whether alone or within a food matrix—undergo sequential steps mimicking the oral, gastric, and intestinal phases of digestion. After digestion is complete, the resulting material can be characterized or applied to in vitro biological systems for toxicity, uptake, or translocation studies.

In conclusion, thorough physico-chemical characterization of the NM, particularly concerning particle size and surface charge, should be considered alongside the selected exposure method to establish and define the sample preparation protocol. Most importantly, to accurately represent real-life exposure scenarios, the properties of the NMs that modulate their cytotoxicity must be preserved in the in vitro setting. For more information on the disadvantages of sonication when preparing NM suspensions, refer to Ruijter et al. (2023).

5.3 Exposure Concentrations

During the innovation process, it is customary—and often expected—to initiate the toxicological assessment of new chemical entities by exposing cells or experimental animals to high concentrations of the chemical. This acute exposure scenario aims to establish the concentrations that elicit positive toxicological responses and to understand the underlying mechanisms of these responses. Despite the importance of acute testing during the innovation phase, most expected exposure scenarios arise from occupational or environmental contexts, where repeated—sometimes continuous—exposure to very low concentrations of the chemical occurs. In this context, the actual concentrations to which humans are exposed in real-life situations are often far below those tested in acute studies, potentially diminishing the relevance of the collected data.

Nevertheless, the disparity between the duration of in vitro tests (typically lasting 24 to 72 hours) and the potential for lifelong, repeated exposure to NMs, may justify testing higher concentrations in the laboratory. Furthermore, the concentrations found in environmental or occupational settings generally exceed those that will reach certain organ systems. For instance, considering the hotspots where inhaled particles interact with the airways—usually at the bifurcations of the respiratory tract (Balásházy et al. 2003)—particle concentrations per surface area may be in the range of μg/cm^2 (Alfaro-Moreno et al. 2010). However, if the evaluated particles impact a secondary target by translocating from the lungs into circulation, plausible particle concentrations may be several orders of magnitude lower.

It is crucial, therefore, to begin testing over a wide concentration range in which the NM is stably dispersed (Swartzwelter et al. 2021), subsequently narrowing down the test concentrations based on the observed results. The extrapolation from in vivo to in vitro remains challenging, not only for NM toxicity testing but also for regular chemical toxicity assessments. Nonetheless, unique challenges may arise for NMs due to their distinct properties, including the biological impact of their physicochemical characteristics and their stability in biological fluids.

Importantly, NMs do not exist in isolation within the environment or in consumer products. Thus, the concept of complex mixtures—comprising other NMs or chemical entities—should be considered when attempting to mimic real-life scenarios in toxicological testing. For further insights and methodologies on complex exposure scenarios, refer to Gerloff et al. (2017).

In conclusion, to obtain relevant safety information, test concentrations should be selected based on the specific scenario being investigated. When aiming to replicate real-life conditions, the expected exposure scenario for the organ of interest should be taken into account, along with data from acute toxicological tests to serve as a reference.

5.4 Dose Metrics for In Vitro Nanomaterial Toxicity Testing

Accurate and relevant dose metrics are essential components of in vitro toxicity testing of NMs. In contrast to bulk materials and chemicals, the mass alone does not sufficiently capture the concentration-effect relationship for NMs. Due to the high surface-to-mass ratio of NMs, expressing their concentration solely in terms of mass concentration (e.g., μg/mL) does not provide all the necessary information for the correct interpretation of the results. This is because adding to cells 200 μL instead of 100 μL of the same concentration of a NP suspension results in double NP number and NP's surface area, which can consequently lead to increased toxicity. Therefore, also surface area (e.g., cm^2/mL) and/or particle number concentration (e.g., particles/mL) should be disclosed (DeLoid et al. 2017; OECD 2012) to avoid misinterpretation of results. However, expressing the dose as a function of volume (e.g., cell culture medium) appears rather indirect and makes it difficult to compare to exposures at the ALI, where the concentration is naturally given as a function of surface area. In in vitro toxicity studies, the cell culture surface area has been proposed as the recommended metric for dose expression (Drasler et al. 2017), e.g., particles/cm^2 cell surface.

It is important to make the distinction between the nominal (i.e., the theoretical) and the effect concentration. This is because, in addition to the NM's soluble components, the cells will mostly interact with the NMs in their close proximity. Thus, in a conventional 2D cell culture, with time, NMs sediment on the cells at the bottom of the plate, which results in an increased effective concentration compared to the nominal concentration (Fig. 2.3). Contrastingly, buoyant NMs never or poorly sediment, lessening their interaction with the cells, and possibly leading to an underestimation of their potential toxicity in a conventional cell culture system (Watson et al. 2016). For such NMs, an inverted cell culture system should be preferred to facilitate the contact between the NM and the cells (for a detailed description see (Watson et al. 2016)). The main mechanisms by which NMs reach the cell surface in in vitro assays are diffusion and sedimentation. These processes are strongly influenced by the size and effective density of the NM. Agglomeration affects key properties like particle size and effective density, which in turn influence the fate and transport of particles in suspension, for example during SUB exposure conditions. The sedimentation rate is proportional to the square of a particle's diameter, resulting in a tenfold increase in size leading to a 100-fold increase in sedimentation rate, and a similar change in the delivered dose (DeLoid et al. 2017). Agglomeration and dispersion may vary with each concentration in the test system, resulting in the total surface area to which cells are exposed not being (proportionally) the same at each concentration (OECD 2012). Higher doses of NM often lead to more agglomeration, which reduces the total number of particles and the total surface area of the NM available to the cells (DeLoid et al. 2017). Multiple mathematical models are available to determine the surface-available exposure concentrations (Cheimarios et al. 2022; Hinderliter et al. 2010; Thomas et al. 2018). Furthermore, NMs may exhibit a dynamic behavior over time (e.g., dissolution, aggregation, and sedimentation), leading to time-dependent changes in the

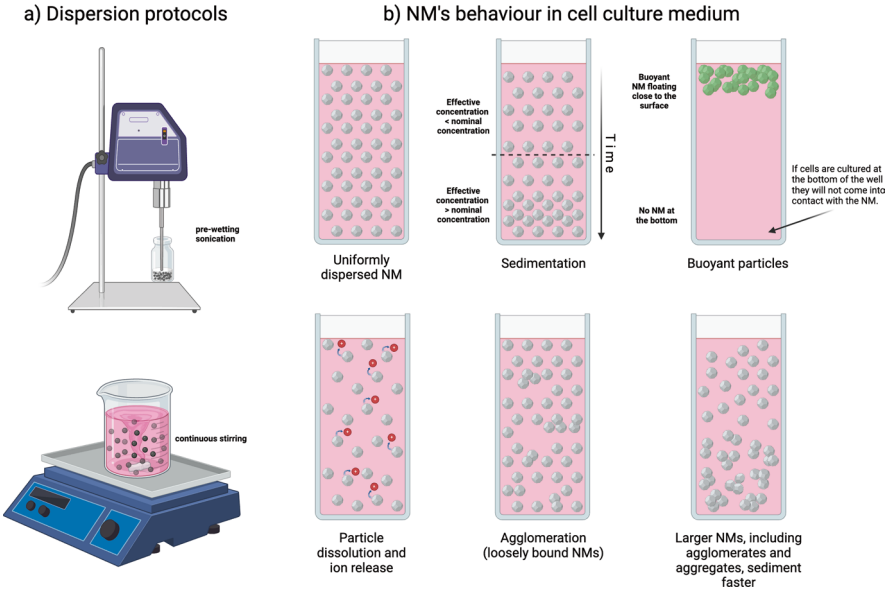


Fig. 2.3 (**a**) Examples of methods used in the preparation of stable NM suspensions. These include pre-wetting and sonication and, more recently, continuous stirring. (**b**) The sedimentation of NMs in the medium/dispersion liquid depends on—for single NMs—the density of the medium, the density of the NMs in the medium, and the diameter of the NM. Intrinsically heavier particles, as well as particle agglomerates and/or aggregates, sediment faster. Buoyant NMs, however, will not sediment at all. Certain NMs are expected to release ions which will most likely be available to interact with the cells

delivered dose. For exposure scenarios longer than 24 hours, it is advised that the composition is checked over time (OECD 2012).

6 Other General Considerations

Considering the similarities in testing between conventional chemicals and NMs, some key aspects apply to both contexts. Here we highlight some of those points:

– Apply the GIVIMP whenever possible: the GIVIMP summarizes the guidelines defined by the OECD as good practice to minimize uncertainties and improve predictions when developing or using in vitro methods (OECD 2018). Among many other points, the document provides guidance on SOP preparation, describes the main factors affecting the reliability and relevance of an in vitro method, and advocates for the importance of establishing definite reporting criteria. It is freely accessible via the OECD website, and it is particularly relevant when seeking regulatory acceptance of in vitro models/methods under development. It is a highly recommended go-to tool for all cell culture users, especially for beginners starting their cell culture routines. Another interesting source of

information on this topic, though more focused on increasing the reproducibility of in vitro models and methods, is the paper (Hirsch and Schildknecht 2019).

- Make sure you have all the necessary materials and reagents in advance, before you start the experiment. Set up a list with all the necessary components for your experiment and take a day or two to prepare all the reagents (except NM suspensions) and cell cultures in advance—this will allow you to maximize results/resources.
- Whenever changes to the procedure are needed, make one change at a time to get the best understanding possible of your model/method/result.
- Start simple and then increase the complexity of both the model and the experimental protocol as you refine your technique and deepen your knowledge about the system.
- Favor the reproducibility of your work by:
  - Using cells from reliable sources.
  - Regularly verifying the authenticity/integrity of your cell lines, testing your cell lines for mycoplasma and, if possible, avoiding the use of antibiotics in your cultures. Besides the generation of resistant bacterial species, the long-term use of antibiotics may induce genetic changes that affect cell proliferation, differentiation, survival, and modify drug response (Weiskirchen et al. 2023).
  - Making sure that environmental conditions remain stable during the experiments. Though many times neglected and even known as "silent variables", environmental conditions, such as temperature, relative humidity, and atmospheric carbon dioxide, may play a significant role in the reproducibility of the observed results. When maintained adequately, cell incubators provide a niche of controlled temperature, CO_2, and humidity levels. When removed from the incubator, even for short periods, cells experience different environmental conditions that can slow down or even halt their metabolism and cell proliferation, such as lower temperature and lower CO_2 levels (which disturb medium pH). Concurrently, the physical movement of the medium on the cells generates some level of shear stress, and some of the medium components are sensitive to white light (OECD 2018). These changes are particularly relevant in models and methods requiring many days in culture or prolonged incubation periods. Adequate incubator maintenance and reducing to the minimum possible the amount of time that cells are outside controlled conditions are crucial factors to consider when performing any in vitro studies (Capes-Davis and Freshney 2021).
  - Preparing detailed SOPs including every step of the experimental procedure is fundamental but many times not enough. An additional one-to-one hands-on knowledge transfer is ideal to make sure all the steps are performed similarly by different operators.
  - Using any automation existing in the lab, namely for cell counting, seeding, exposure, and sampling. This will greatly reduce errors associated with the operator. These and other suggestions to improve the reproducibility of

in vitro studies can be found in (Capes-Davis and Freshney 2021; Hirsch and Schildknecht 2019).

- Try to collect as much information as possible from the same in vitro model unit (well, chip, insert, etc.) (Drasler et al. 2017). Multiplexing-compatible assays are a great way to see the bigger picture and provide more power to your assumptions. Nowadays, there are many available non-destructible techniques that can be used in tandem to generate meaningful information about the status of the in vitro cultures and how the tested item affects their health. Supernatants are a great source of information on what is happening in the cell population throughout time in culture, and their analysis in multiplexed systems may be a great alternative to replace destructive methods.

7 Conclusions and Perspectives

While the field of nanotoxicology has benefited from the tradition and knowledge of conventional toxicology, the assessment of the safety of NMs comes with unprecedented challenges, which make a thorough and thoughtful experimental design a real must. The recent investments in the development of new human-relevant advanced in vitro models allowing new routes of exposure open new avenues for recreating closer-to-real-life environments in the lab. Prior investigation and reflection about possible exposure scenarios (route, NM concentrations, etc.) will help define the proper in vitro models and test methods to provide the most relevant data on the effects and mechanisms triggered by NMs.

New and exciting developments in methodological assessments have been observed in recent years, granting access to big data at the cellular population and/or single-cell level. Therefore, many of the conditions seem to be in place to make the most out of both models and methods to advance the state of the art of human-relevant nanotoxicology research.

With these new technologies come new needs for collaborative efforts in harmonization and development of guidelines for best practices. Even though many efforts have already been undertaken in this regard, leading to the generation of valuable standardization protocols, there is substantially more new knowledge that needs to be integrated into these protocols and further disseminated. Consistency in methodologies, especially for characterization, sample preparation, and exposure scenarios, is crucial to enhance the reproducibility and comparability of results across different laboratories.

Particularly, harmonization of exposure methods using generation of aerosols at the ALI would greatly benefit the scientific community. Because the *dose* still *makes the poison*, precise dosimetry must be implemented by means of reliable recording of the actual dose deposited over time and its distribution across the cell layer, together with accurate monitoring of environmental conditions (e.g., humidity and temperature). If widely and openly shared and applied, all these protocols would jointly contribute to advance our understanding of the detrimental effects that NMs may (or may not) pose to human health and the environment.

Acknowledgements This work was funded by the European Union's H2020 project Sinfonia (N.857253) and PHOENIX (N.953110), as well as by Horizon Europe Projects LEARN (N.101057510) and iCare (N.101092971). FC is supported by the Portuguese Foundation for Science and Technology (FCT) through projects UIDP/04378/2020 and UIDB/04378/2020 (UCIBIO), and LA/P/0140/2020 (i4HB).

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