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Review

Hazard assessment of nanomaterials using *in vitro* toxicity assays: Guidance on potential assay interferences and mitigating actions to avoid biased results

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ABSTRACT

The movement towards an animal-free testing approach for risk assessment represents a key paradigm shift in toxicology. Risk assessment of engineered and anthropogenic nanoscale materials (NM) is dependent on reliable hazard characterization, which requires validated test methods and models, and increasingly on mechanistic insights into the mode of action. The properties that make NMs so advantageous for a wide range of commercial and industrial applications also pose a challenge when it comes to safety testing under *in vitro* and *in chemico* experimental settings. Their large reactive surface area makes NMs prone to interactions with assay reagents, readout signals, or intermediate steps of many test assays, leading to the potential for biased results and data inconsistencies, collectively referred to as interferences. Therefore, methods and protocols developed and validated for conventional chemicals often require adaptation and checking for reliability in NMs' toxicity assessment. This review presents the collected scientific knowledge on NMs-induced interferences for the most common *in vitro* toxicity assays and methods related to cytotoxicity, oxidative stress and inflammatory response evaluation. Our analysis of existing scientific literature showed that the challenge of NMs-induced interference was not explicitly addressed in more than 90% of the papers published up to 2014 reporting the safety and toxicity of NMs. In later years, increasing number of studies tackled the interference challenge in toxicity testing of NMs, which initiated exhaustive work on standardization and validation of existing regulatory-relevant *in vitro* test protocols and guidelines. Due to the specificity of the different NMs and the range of ways they can potentially interfere with *in vitro* assays, interference and fit-for purpose controls should be included for each NM type and method applied, unless label-free assays are selected. Here, we provide a decision tree to guide researchers on how to design experiments to avoid interferences during *in vitro* testing by taking appropriate mitigation actions and how to include proper interference controls in their experimental design where complete avoidance is not possible. The application of this decision tree will improve the reliability, comparability and reusability of *in vitro* toxicity data on engineered NMs or ENMs, increasing the relevance of *in silico* hazard data for use in risk assessment and in science-based risk governance of NMs. The approach is applicable more broadly

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also, to advanced materials and to hazard assessment of anthropogenic nanoscale materials such as microplastic and tyre-wear particles.

Introduction

The 3Rs directive (Directive 2010/63/EU), to replace, reduce and refine animal experiments, is driving efforts to move from *in vivo* studies towards *in vitro* and *in silico* approaches. Standardized and validated *in vitro* test methods and models are essential to this transition and are especially important for safety assessment of nanomaterials (NMs), defined as materials with at least one dimension less than 100 nm [1], as their toxicity behaviour often deviates from that of their bulk counterparts. Due to their particulate nature and nanoscale dimensions, NMs behaviour does not always follow that of other materials or chemicals. For example, as their size decreases, their surface area per mass unit increases, enhancing their reactivity and adsorption capacity. This makes NMs prone to aggregation, agglomeration and/or interaction with other molecules as a mean of passivating the nanosurface. Such NMs behaviour has been shown to make them prone to interfering with optical readouts and assay reagents [2] or to induce secondary toxicity by binding of available biomolecules (nutrients) thus leading to cell starvation [3]. Thus, the unique properties of NMs need particular consideration when selecting *in vitro* toxicity assays since there are many well-documented cases of specific NMs interfering with different assays and techniques [1,3,4] as summarised in Table S1 in the supplementary information. However, systematic reporting of checks and controls for NMs-induced interferences with toxicological test methods and test guidelines (TGs) is scarce despite growing awareness of the issues. Some authors did not provide sufficient information or data to rule out potential interferences or did not systematically investigate interferences, other authors did not even consider the possibility of interferences being induced by NMs during testing, while a third category of others assume that these issues were addressed previously and are no longer an issue despite the constantly evolving complexity of NMs and the continual turnover of researchers performing such assays.

Almost a decade ago, review by Ong et al. [5] highlighted that more than 90% of the scientific papers published in the period from 2012 to 2014 on the use of colorimetric assays to test the NMs toxicity neither considered nor included interference controls. Since then, interference challenges have been increasingly addressed during NMs toxicity evaluation, especially as part of the efforts to standardize and validate existing test protocols and OECD TGs for hazard assessment of NMs, for instance in the European FP7 project NanoTEST, and the H2020-funded projects NanoREG, MARINA and especially RiskGONE which had a strong focus on validation of label-free and high throughput methods. One of the main aims of the RiskGONE project was to evaluate the suitability of various *in vitro* test protocols for reliable safety assessment of NMs, and to deliver sound regulatory relevant and science-based approaches for risk assessment of NMs. This is considered essential to facilitate the paradigm shift based on the development of new (animal-free) alternative methodologies (NAMs) including advanced cellular models [6].

This review compiles scientific data available on NMs-induced interferences with *in vitro* assays from the last ten years, highlighting the critical steps where NMs-induced interferences may occur and provides mitigation actions to avoid them. Assays for the most common biological endpoints including cytotoxicity, oxidative stress response, inflammation and protein expression / protein content were selected for review. These assays are summarized and grouped by endpoint, while relevant approaches and positive/negative controls to assess for interference are also described. Where NMs-induced interferences are identified, appropriate mitigation actions are suggested, or where mitigation is not possible alternative assay suggestions are provided. To support the users in the design of assays, a series of recommendations related to each endpoint are summarised into a decision tree to guide users through the selection of appropriate controls, mitigation strategies or alternative assays in order to overcome any potential interferences. We note also that not every type/composition of NM induces every potential interference (see Table S1 for a comprehensive list of the NMs reported to induce specific interferences). Thus, our ongoing work aims to integrate NM-specific recommendations into a more robust decision tree that includes also details on the NM composition and type as well as the assay type.

Materials and methods

This literature review collected and evaluated all data from peer-reviewed papers published in the period from 2014 and 2022 on NMs-induced interferences with *in vitro* assays, specifically focusing on assays based on colorimetric, fluorometric and luminescence readouts, excluding genotoxicity assays which are reviewed elsewhere. The literature search was carried out in the PubMed database using "interference", "nano*" and "in vitro" as keywords. The last search was conducted on December 8th, 2022. No filters or language restrictions were initially applied resulting in 10,029 articles hits. These articles were screened for relevance related to the selected toxicological endpoints (i.e., cytotoxicity, oxidative stress response, inflammation and protein quantification), which finally yielded only 73 articles. The articles were subjected to quality evaluation and data extraction following recommendations for best practices in nanotoxicology research using the approach developed under the GUIDEnano project [7]. Briefly, the approach is based on the use of K and S scores: the K score is related to test design and follows the principles and considerations of the ToxR-Tool [8]. The S score is based on the reported list of physicochemical properties of NMs that should be characterized in the exposure medium [9].

Results of the literature analysis on NMs interference with *in vitro* assays

Our literature analysis, focused on the toxicological endpoints of cytotoxicity, oxidative stress response, inflammation and protein quantification, revealed several causes of NMs-induced interferences with the *in vitro* assays. These include optical quenching, autofluorescence, interactions of NMs with the test reagents, analytes, and/or reaction products at different steps of the assay. In order to fully understand the source of the interference, to identify where possible interference may occur during nanotoxicity testing and what mitigation actions should be taken, it is crucial to consider both the physicochemical characteristics of NMs and the underlying principle of each assay. In the following sections, the findings and a discussion of the mitigation measures and potential alternative assays for each selected toxicological endpoint are described.

Cytotoxicity

When performing safety assessment of NMs, cytotoxicity is one of the first investigated endpoints and often used for screening purposes to provide a concentration response for *in vitro* cell exposure to NMs, which is then a basis for the evaluation of other toxic endpoints. Cytotoxicity can be determined by employing different biomarkers, such as cell death and/or cell viability, through measurement of cell proliferation, cellular metabolic activity, lysosomal//mitochondrial activity or cell membrane permeability. There are a wide range of assays to study cytotoxicity, most of which rely on fluorometric or colorimetric measurements.

Table 1
Cytotoxicity assays: potential interferences from nanomaterials.

Assays	Reagents	Readout parameters	Type of interference	References
Cytotoxicity				
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide (MTT)	absorbance (590 nm)	Light absorption	[10,32,33,35,104–107]
			Adsorption of reagents	[105,107,108]
			Electron transfer from reagents to graphene p-p orbitals	[106]
			Induction of formazan generation	[109]
			Production of measurable end-products	[105]
MTS	3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium (MTS)	absorbance (490 nm)	Light absorption	[33,107,110]
			Binding to the assay components	[5,107]
LDH	2-(4-iodophenyl)-3-(4-nitrophenyl)-5-phenyl tetrazolium chloride (INT)	absorbance (492 nm)	Light absorption	[5,35,104]
			Adsorption of reagents	[109,111,112]
			Inactivation of reagents	[111,112].
			Interference with the reagent	[113]
Neutral red	N^2,N^2,7-trimethylphenazine-2,8-diamine (neutral red; NR)	absorbance (550 nm)	Reaction with assay components	[32,105]
			Particle solvent affects the results	[31]
			Production of measurable end-products	[105]
			Light absorption	[10,105]
CytoTox One	CytoTox One™ kit	fluorescence; excitation (560 nm), emission (590 nm)	Dye oxidation	[32]
Flow cytometry	Annexin V-FITC, propidium iodide (PI)	fluorescence; excitation (490 nm, Annexin V; 535 nm; PI), emission (525 nm, Annexin V; 617 nm, PI)	Sticking to the cell's surface changing fluorescence	[23]
			NMs adsorbing dye	
			Quenching fluorescence	
WST-1	2-(4-iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2*H*-tetrazolium,	absorbance (450 nm)	Interference with the conversion of WST-1 into formazan	[114]
	monosodium salt (WST-1)		Light absorption	[10]
WST-8	2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-	absorbance (450 nm)	Light absorption	[33,106]
(CCK-8)	2*H*-tetrazolium, sodium salt (WST-8)		Electron transfer from reagents to graphene p-p orbitals	[106]
			Solvent in which NMs are dispersed affects results	[31]
			Adsorption of reagents	[106]
			Blocks development of WST-8 color	[113]
Live/Dead assay	Calcein AM (CAM), ethidium homodimer-1 (EthD-1)	fluorescence; excitation (488 nm), emission (530 nm, CAM; 610 nm, EthD-1)	Reagent adsorption	[110]
			NM agglomerates blocked fluorescence	[32]
CellTiter Blue	CellTiter-Blue™ Reagent	absorbance (590 nm)	Fluorescent quenching	[32]
			NM agglomerates blocked fluorescence	[31]
Acridine and coumarin staining	acridinium derivatives, aminocoumarin	fluorescence; excitation (480 nm), emission (520 nm)	Quenching of the fluorescence	[115]
Alamar Blue	Alamar Blue™	fluorescence; excitation (530–560 nm), emission (590 nm)	Alteration of oxidative state of the dye	[5,32]
Propidium iodide	propidium iodide (PI)	fluorescence; excitation (535 nm), emission (617 nm)	NM agglomerates mistaken for cells	[10]
Trypan blue	trypan blue	Cell counting	NM agglomerates mistaken for cells	
Cell proliferation assay/Proliferative activity of peripheral blood cells				
^{3}H-Thymidine incorporation	^{3}H-thymidine	radioactivity counts	Reduction of iron oxide NMs	[10,116]
			Adsorption of emitted electrons by NM agglomerates	[10]
			Optical quenching	
BrdU	bromodeoxyuridine (BrdU)	absorbance (450 nm)	NM agglomerates block fluorescence	
Apoptosis				
Annexin V	Labelled-Annexin V, DNA dyes	fluorescence	SCS versus FCS and fluorescence modulation, Adsorption of the probe	[15,23]

(*continued on next page*)

Table 1 (*continued*)

Assays	Reagents	Readout parameters	Type of interference	References
DNA fragmentation	DNA dyes	fluorescence	SCS versus FCS and fluorescence modulation Adsorption of the probe	[15]
Cell death detection ELISA	ELISA kit	absorbance	Non-defined	[15]
Caspase assay	Peptide/aminoluciferin	luminescence	Interference with recovery of the analyte	[15]
Mitochondrial transmembrane potential	JC-1	fluorescence	Fluorescence quenching, adsorption, Aggregation of the probe	[34]

Table 1 summarizes the results from our literature search on NMs interferences with cytotoxicity test methods. It is worth mentioning that this is a non-exhaustive list of assays as it is based on the assays/tests for which NMs-induced interferences were reported. For each assay presented in Table 1, we identified the critical steps where NMs-induced interference may occur.

Assays for determining cell viability or cell death are generally based on the staining of live or dying/dead cells. One of the simplest assays to assess cell death is cell counting using trypan blue dye which stains dead cells, while live cells stay non-stained [10]. This assay is not based on fluorometric or colorimetric readouts, but on a simple counting of stained cells using light microscopy. Cell viability can also be evaluated by measuring the activity of lactate dehydrogenase (LDH), which is released into the extracellular medium after cell death. LDH activity is usually measured by a colorimetric/fluorometric assay. Many other assays for cell viability or cell death are fluorometric-based, like those for studying apoptosis or programmed cell death, which is generally characterized by distinct morphological characteristics and energy-dependent biochemical mechanisms [11,12]. One of the most widely used approaches is the annexin V and propidium iodide (PI) double staining assay coupled with flow cytometry [13]. Likewise, the luminescent-based caspase assay is based on analysis of the activation of different families of caspases that are critically involved in the apoptotic process [14,15]. The analysis of caspase activation is often used to study apoptosis by immunostaining coupled with image analysis or flow cytometry [16]. Other fluorescence-based assays use fluorescent probes like JC-1 or MitoTracker for analysis of changes in mitochondrial transmembrane potential [17,18], intercalating DNA dyes such as PI or TdT-mediated dUTP nick end labelling (TUNEL) assay for detecting DNA fragmentation [19]. In the TUNEL assay, labelled dUTP is incorporated by the enzyme terminal deoxynucleotidyl transferase into free 3′-hydroxyl termini generated by the fragmentation of DNA and the incorporated dUTPs are subsequently detected by various (colorimetric or fluorometric) methods [20,21]. Interference problems with these assays were reported for various NMs depending on the NMs' light scattering and/or fluorescence properties and/or their ability to interact with fluorophores, which in turn can depend on their agglomeration state and colloidal stability in the exposure medium [15,22,23].

Metabolic activity is another common biological property used to evaluate cell viability using assays such as Alamar Blue (AB), the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2 H-tetrazolium bromide (MTT), the water-soluble tetrazolium salt (WST-1) and the 2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2 H-tetrazolium-5-carboxanilide (XTT), which are based on metabolic conversion of a reagent to a product that can be measured by absorbance or fluorescence readings (as either loss of reagent or gain of product). The ability of NMs to interfere with these assays has been well described [10,24]. NMs-induced interference with assays evaluating cell proliferation has also been reported for Thymidine incorporation assay, BrdU assay, and DAPI or Hoechst staining. The Thymidine incorporation assay measures cell proliferation by quantification of radio-labelled thymidine incorporation into nuclear DNA during the S phase of the cell cycle [25], while the BrdU assay is based on incorporation of synthetic nucleoside analogues of thymidine into DNA during the S-phase and quantification of the analogues using monoclonal antibodies followed by counting of labelled cells or enzyme-linked immunosorbent assay (ELISA) [26]. DAPI and Hoechst are DNA intercalating dyes which become fluorescent when bound to DNA and are classically used to stain cell nuclei for microscopical cell counting, and for measuring cell proliferation [27,28]. Interference with DAPI and Hoechst dyes are of particular interest for NMs' hazard assessment since these dyes are commonly used in high-throughput (HTP) techniques for the identification of cell nuclei [29]. Interference of NMs with cell count staining techniques has been demonstrated both for absorbance and fluorescence measurements, by absorption or quenching of signals at a particular wavelength, as well as through quenching of beta-counts [30,31].

Interferences with the above-mentioned assays have been reported for many different types of NMs. For example, carbon-based NMs, such as carbon black (CB), fullerene, graphene, single-or multi-walled carbon nanotubes (SWCNTs / MWCNTs) can interfere with cell counting if the NMs or their agglomerates/aggregates are recognised (counted) as cells by the counting devices used [32]. Furthermore, carbon NMs may cause dye oxidation (*e.g.*, in the CytoToxOne assay), reduction (*e.g.*, in the Alamar Blue assay), and blocking or quenching of the fluorescence (*e.g.*, in Calcein/EthD-1 staining) [32]. In the LDH, MTT or WST-8 assays, carbon-based NMs may interfere with light absorbance, by adsorbing the reagent or blocking the development of the reaction product [32]. Similar types of interferences as reported for carbon-based NMs were also reported for metal-based NMs including Ag-NMs, Fe-NMs [33], FeO-NMs, AgO-NMs, CoO-NMs, CeO_2-NMs, Au-NMs [31,34], and AlOOH-NMs, Al-Ti-Zr-NMs, $SrCO_3$-NMs, Ti-Zr NMs, ZrO_2 -NMs, $BaSO_4$ -NMs, and ZnO-NMs [35]. Most of these studies demonstrated that the extent and the nature of interferences were dependent on the physico-chemical properties of the NMs, such as particle core composition and surface functionalisation [10,15,36,37].

Finally, each interference problem mentioned for the different cytotoxicity assays are significantly affected by the agglomeration state and colloidal stability of the tested NMs as these properties determine also the specific surface area available for adsorption and the optical properties for each NM type.

Oxidative stress

Oxidative stress in living cells and tissues is a consequence of the imbalance of oxidant and antioxidant processes, when the increased production of reactive oxygen and/or nitrogen species (ROS/RNS) may damage proteins, lipids, and genetic material (DNA). Indeed, oxidative stress has been described as a key event in health-related adverse outcomes such as cancer, neurodegenerative disorders, and atherosclerosis [38,39]. Moreover, it has been considered as one of the major and most common mechanisms underlying NMs toxicity [40,41]. The extent of oxidative stress can be evaluated either by measuring free radicals (*e.g.*, ROS), the products of oxidative stress (*e.g.* protein carbonylation or lipid peroxidation) or by assessing the changes in the antioxidant capacity of cells (*e.g.*, activity of antioxidant enzymes or concentration of antioxidants). Table 2 lists the reported interferences and their causes for

Table 2
Oxidative stress assays: potential interferences from nanomaterials.

Assay	Reagents	Readout parameters	Type of interference	Reference
Oxidative stress				
DCFH-DA	2,7-	fluorescence; excitation	fluorescence quenching	[10,45,104, 117]
	dichlorodihydrofluorescein diacetate (H_2DCF-DA)	(485 nm), emission (520 nm)	dye oxidation	[33,110]
			NMs background fluorescence	[33]
			light scattering	[35]
			increase of fluorescence	[10]
DHE	dihydroethidium (DHE)	fluorescence; excitation (485 nm) emission (570 nm)	fluorescence quenching	[33]
mBCl	monochlorobimane (mBCl)	fluorescence; excitation (355 nm) emission (460 nm)	fluorescence quenching	
mBBr	monobromobimane (mBBr)	fluorescence; excitation (360 nm) emission (460 nm)	quenching or increase of the fluorescence	[10]
APF	3'-(*p*-aminophenyl) fluorescein (APF)	fluorescence, excitation (480 nm), emission (520 nm)	dye oxidation	[110]
Catalase activity/Antioxidative systems				
Amplex red catalase assay	Amplex Red Catalase Assay Kit	absorbance (560 nm); fluorescence, excitation (535 nm), emission (595 nm)	Alteration to oxidative state of the dye	[5]

different *in vitro* assays designed to evaluate the oxidative stress response.

As with the dye-based cytotoxicity assays, NMs-induced interferences have been reported for many of the oxidative stress assays using fluorescent probes such as dichlorofluorescein (DCFH), rhodamine 123 (Rh123) and dyhydroethidium (DHE), which are oxidised into fluorescent compounds in the presence of ROS, that can be measured by spectrofluorimetry, with the amount of fluorescence being proportional to the amount of ROS present in the sample [42]. In acellular experiments, the DCFH probe is widely used to analyse the intrinsic ability of NMs either to generate ROS and/or to deplete antioxidants. Since these assays rely on fluorescence measurement, it is important to check if the specific NM can enhance or inhibit the fluorescent signal, or even interact with reagents or reaction products, similar to what has been reported for cytotoxicity assays [43]. A recent review of ROS formation by metal and metal oxide NMs in physiological media identified the reactions that may occur in solution as these can vary considerably depending on the chemical setting [44]. The authors suggest that any direct comparison between different solutions is questionable without knowledge on metal speciation since metal complexation can influence ROS formation [44].

Table 2 lists reported evidence for NMs-induced interferences with methods for oxidative stress evaluation (Table 2). For example, fluorescent signals were reduced by CB and TiO_2 NMs during DCFH measurement, while interference with the DCF product was observed with different metal-based NMs such as AgNMs, CeO_2-NMs, AlOH-NMs, Ti-Zr-NMs, TiAlZr-NMs, ZrO_2-NMs, $BaSO_4$-NMs, Fe_3O_4-NMs, $SrCO_3$-NMs, ZnO-NMs [10,33,35] and carbon-based NMs [45]. Interferences were also observed with other assays which employ fluorescent dyes as marker for ROS such as the Dihydroethidium (DHE) assay, and for measuring glutathione levels such as the monochlorobimane (mBCl) assay [33]. These interferences occurred either by fluorescence quenching, dye oxidation or dye adsorption, which is enhanced with increasing NM concentration [33]. It is important to highlight that the interference observed depends on the physico-chemical characteristics of the NMs like surface coating as observed in the case of Ag-NMs [33]. Similar interferences were observed between TiO_2 -NMs and helical rosette nanotubes (RNT) using the catalase assay [5]. The TiO_2-NMs showed interference with this assay even in the absence of the catalase, thus interfering with either the fluorescence or absorbance readouts [5].

To evaluate potential interferences of NMs with oxidative stress assays, it has been suggested to incubate the NMs with specific ROS generating systems, such as xanthine/xanthine oxidase or H_2O_2/Fe [43]. Use of more biologically or environmentally realistic media containing serum or other proteins has also been suggested to ameliorate interferences due to binding of analytes to NM surfaces, but the potential for overestimating the fluorescence / absorbance must also be assessed [5].

Inflammatory responses

Inflammatory responses, including release of cytokines, have been highlighted as another mechanism of NMs toxicity [46–48]. Cytokine release is typically analysed in the cell culture medium through ELISA, a so called "sandwich" assay in which a primary antibody is adsorbed to the surface of a high-affinity binding microwell-plate which recognizes and binds the protein of interest in the cell culture supernatant, and then a secondary biotinylated antibody binds to the same protein of interest, but at a different epitope, which serves as the detection antibody [49]. NMs can interfere with ELISA as a result of different phenomena such as intrinsic NMs catalytic activity, scattering/absorbance properties, and binding of antibodies or antigens, as shown schematically in Fig. 1 from Hirsch (procedure for assessment of NMs Interference in ELISA) [49].

In addition to optical interferences, adsorption of cytokines onto NM surfaces has been reported, which blocks their interaction with the anti-cytokine antibodies used in the assay [50]. As summarized in Table 3, such interferences are dependent on the nature and concentration of the cytokines as well as on the nature and concentration of the NMs, which in turn determines the NMs surface area available for binding, but also influences the potential for NMs agglomeration [10,35,51–54].

Besides cytokines, endotoxins, heat-stable lipopolysaccharides found on the outer cell wall of gram-negative bacteria, are well-known to induce inflammation. The presence and the amount of endotoxin associated with NMs are typically measured using the Limulus Ameobocyte Lysate Assay (LAL) assay which quantifies the endotoxin concentration [55]. Due to their high surface adsorption capacity, NMs are well-known to be easily contaminated with endotoxins during the preparation process, and thus when measuring NMs-induced inflammation it is essential to test for, and rule out, endotoxin contamination as the source of any inflammation observed with NMs exposure. This contamination can occur at any stage of NMs production or handling and is difficult to prevent or remove due to the thermostability of endotoxins, which are resistant towards most standard sterilization methods applied in biological laboratories [56]. While there are techniques for sterilisation of NMs, such as filtration, autoclaving and irradiation, formaldehyde, ethylene oxide and gas plasma treatments, no single process may be applied to all NM-types without causing some alterations of the NMs physicochemical properties, thus also affecting their toxicity and functionality [57]. Since endotoxin presence is known to influence product safety and efficacy, evaluation of endotoxin contamination is also considered as critical issue in safety assessment of NMs [58,59]. LAL assay can be performed by using turbidimetry or it can be chromo/-fluorogenic. However, various NMs are known to interfere with this assay (Table 3) either due to their optical properties (absorbance, fluorescence, turbidity) or due to binding of reagent/analyte onto the NMs surface [58,60–63]. In addition, interferences with the LAL assay depend on the NMs type and concentration, nanosurface

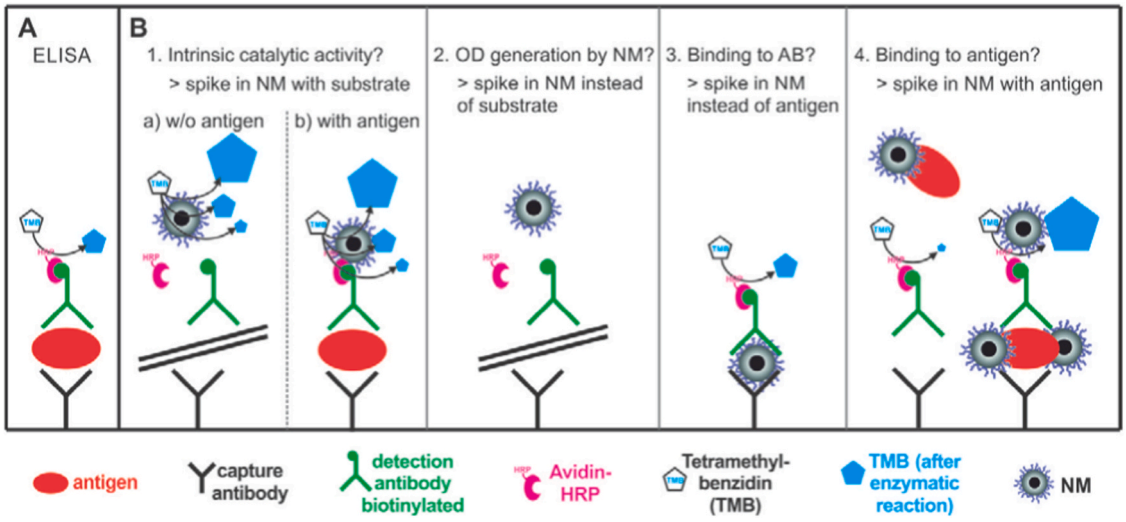

Fig. 1. Schematic overview of potential NM interference sites during an ELISA procedure. A) the sandwich assay; B) the 4 major types of potential interference from NMs: 1. NMs that possess intrinsic catalytic activity may process the substrate themselves; 2. the presence of NMs may change the optical density (OD) of the solution; 3. NMs may bind to the antibodies used resulting in a false positive signal; and 4. NMs may bind to the antigen, thus preventing the antigen from binding to the antibody causing a false negative result, or increase the antigen affinity towards the antibodies causing a false positive result. From the procedure for assessment of NMs Interference in ELISA by Hirsch [49].

Table 3
Other relevant endpoints (inflammation, pro-inflammation and protein binding) and the potential interferences from nanomaterials.

Assay	Reagents	Reading parameters	Type of interference	Reference
Pro-inflammatory response				
Cytokine measurment (ELISA kit)	ELISA kit	absorbance (450 nm)	Cytokine adsorption	[10,35, 50–54]
LAL assay: gel clot	LAL, coagulogen protein	clot formation	Interaction with proteins	[58,61,62]
LAL assay: chromogenic	LAL, p-nitroaniline	absorbance (450 nm)	Interference with readout, interaction with endotoxin or with reagents	[58,62,63]
LAL assay: turbidimetric	LAL	turbidimetry	Intrinsic turbidity	[58]
TLR4 reporter cells	Reporter gene expression	absorbance, fluorescence	Interference with reagents and/or readout	[62]
MAT (ELISA kit)	ELISA kit	Absorbance (450 nm)	Interference with reagents and/or readout; Interaction with proteins; Incomplete recovery of proteins	[63]
HPLC-MS	MS	2-hydroxy and 3-hydroxy fatty acid	Absence of NMs interferences reported	[81]
Protein quantification / Protein corona considerations				
BCA assay	Bicinchoninic acid (BCA)	absorbance (562 nm)	dye reduction	[5,118]
Bradford protein assay	Coomassie Brilliant Blue G-250 dye	absorbance (595 nm)	binding to the assay components	

functionalization [61] and on the format of this assay [31,58]. For example, SiO_2, TiO_2, Ag, and $CaCO_3$ NMs interfered with gel clot LAL assay, while no interference was observed with the chromogenic-based LAL assay [61]. Dobrovolskaia et al., suggested to run different formats of the LAL assay to ensure that endotoxin results are consistent and not impacted by NMs-induced interference [58].

Protein quantification

Assays for protein quantification are commonly used to normalise experimental data on the basis of the protein concentration in the sample. The most common are the Bicinchoninic Acid (BCA) assay and the Bradford assay [64]. As these assays are based on optical readouts, NMs can interfere with them in all the same ways as noted above for cytotoxicity and cytokine assays, i.e., by changing the scattering or absorbance of the solution or by binding the proteins and thereby reducing their detection. Another source of interference may be the use of the Laemmli buffer for denaturation and extraction of proteins [65]. The Laemmli buffer may interfere with the downstream protein quantification methods (*e.g.*, Bradford assay) due to the high amount of detergent used (typically 2% sodium dodecyl sulphate (SDS)) and a possible solution to this is to precipitate the proteins using for example ice-cold acetone/trichloroacetic acid (TCA) solution prior to the redissolution of the pellet in buffer, although it must be noted that this is a method for removal of albumin from serum [66] and thus will influence the results if being used to assess NMs corona composition [67] or through the use of other quantification methods like the BCA assay, which is a detergent-compatible formulation (due to the extensive dilution) and relies on the Biuret reaction (reduction of Cu^{2+} to Cu^{1+} by the peptide bond in alkaline conditions). However, the presence of ions released from NMs could potentially interfere with the redox-reaction and lead to over/under estimation of the amounts of proteins present/released. In addition, the phenomenon of protein adsorption onto NMs surfaces is another source of possible interference during protein quantification. There are only few reports on NMs-induced interference with these methods, e.g., for the SWCNT, Si and CdSe NMs with the Bradford assay [5] or for iron oxide NMs with the BCA assay [68].

Biomolecular corona formation on NMs surfaces may also induce interference with a range of different assays. For example, bovine serum albumin (BSA) is one of the widely used blocking agents in several protein binding assays such as western blotting where it quenches non-specific adsorption and binding of proteins to tubes surfaces and pipette

Table 4
Description of the mitigation measures as described by the first decision tree for addressing NMs-induced interferences with in vitro toxicological test.

Type of interference	Test(s) to identify / rule out specific interference	Potential mitigating measures	Alternative tests if mitigation not possible
NMs interfere with assay readout	• Spectroscopic analysis (absorbance, fluorescence, luminescence) of all assay components and NMs in acellular system at different doses • Check for increased signal in treated cells by omitting reagent	• Subtract background signal • Perform additional washes/ centrifugation to eliminate interfering NMs • Work at concentrations free of interferences • Choose another assay with a different readout • Choose a label-free assay	• For cytotoxicity: • Colony Forming Efficiency assay • Impedance-based assays • For oxidative stress: • electron spin resonance (ESR) / electron paramagnetic resonance (EPR) spectroscopy • indirect evaluation of non-enzymatic anti-oxidants (cellular thiols) or enzymes involved in antioxidative defence systems • two-photon fluorescence lifetime imaging microscopy (FLIM)
NMs interfere with reagent, analyte or reaction of interest	• Perform spiking / recovery analysis of the reagent and analyte in acellular system • Assess whether more realistic medium ameliorates effect (e.g., BSA or serum proteins to coat NMs) • Assess surface speciation of the NMs / catalytic activity • Check binding interactions of reagent/analyte with NMs • Check interactions of NMs with assay reaction by using positive control with/ without NMs addition • Check for endotoxin contamination	• Dilute the sample • Perform additional washes / centrifugation to eliminate interfering NMs • Work at concentrations free of interferences • Choose alternative dyes/fluorescent probes • Choose alternative assay (*e.g.*, label-free assay)	• For inflammatory responses: • impedance-based and label-free assays
Interferences with flow cytometry based assays	• Check modulation of optical properties of the fluorophore with NM spiking controls in untreated cells • Exclude particles from analysis by gating (using SCS versus FCS with a NM only sample) • Include spike-in experiments applicable to all NM / cell types	• Select another assay using alternative dyes/fluorescent probes • Perform additional washes/ centrifugation to eliminate interfering NMs • Work at concentrations free of interferences • Select alternative assay / choose a label-free assay	Impedance-based flow cytometry (IFC)
Protein binding considerations	• Interference with denaturation solutions during extraction of proteins for BCA/Bradford assay • Check binding of proteins (*e.g.*, cytokines) of interest to NMs and if reduced with pre-formed corona • Include at least two empty tubes to determine potential contamination of LPS before and during sample preparation • Where binding to NMs is desired, check recovery / desorption method used to ensure complete recovery	• Apply another denaturing approach / buffer • Use blocking agent, e.g., BSA or ovalbumin to "block" binding sites • Explore different methods of recovery of proteins, and impacts of different choices on total protein detection	Ultra- high performance liquid chromatography coupled with mass spectrometry (HPLC-MS) Apply on-particle digestion approaches (*e.g.*, [82]

tips [69]. Thus, this blocking or surface passivating role of BSA may be disrupted by protein corona formation on NMs as already well evidenced for many different NM compositions [70,71]. We note that BSA and human serum albumin (HSA) share a sequence similarity of 77.5%, yet have been shown to interact slightly differently with 30 nm gold NMs as investigated by surface-enhanced Raman scattering, with both proteins binding electrostatically to citrate ligands on the NMs *via* lysine residues, with HSA also binding directly to the gold surface by particularly flexible protein segments that were identified by comparison of the vibrational bands with the known amino acid sequence of the molecule [72]. BSA accounts for about 95% of the protein content in FBS which is widely used as the nutrient source for cell culture even with human cells. The NMs coronas formed in human or foetal bovine serum have been found to comprise many homologous proteins, but to lead to different NMs toxicity in HepG2 cells with the human serum corona NMs being 4-fold less toxic than FBS-corona NMs, likely as a result of "markers of self" being present in the human serum that are recognized by human cell receptors [73].

Recommendations and mitigation actions

Mitigation through tweaking experimental conditions

Based on the data collected and presented in Tables 1–3, it is clear that all possible NMs-induced interferences should be considered during *in vitro* safety testing of NMs, while inclusion of appropriate controls is essential during the experimental design. Understanding the range of potential NMs-induced assay interferences is extremely important during experimental data collection and aggregation of NMs toxicity data for further meta-analysis and modelling purposes. Furthermore, correct planning for potential interferences will reduce the errors or biases in future studies.

In general, assessment of each toxicological endpoint should be performed through a combination of different assays, with each relying on different measurement principles and with appropriate interference controls (see Table 4) for each test method applied, in order to avoid misinterpretation and to strengthen the conclusions that can be drawn from the experimental data [32,43]. Interference can lead to both over- and under-estimation of the NMs toxicity. Thus, tests for interferences should always be integrated in *in vitro* hazard identification of NMs. However, such tests and controls are often time-consuming, complex

and not always easy to set up, requiring expert knowledge of the assay principles to carefully design them. For example, a check for potential interferences with the assay readouts, the reagents, the analyte and the reaction product [34] should be completed, as well as for NM reactivity (*e.g.*, catalytic activity, changes in surface speciation, a shielding role of any pre-formed protein corona etc.) especially under the realistic exposure medium conditions. The experimental design gets even more complicated when multiple interferences are occurring during an assay [34] or when different NMs interfere with the same assay by different modalities. Some interference issues are easy to overcome with small changes in the experimental conditions, while others might be impossible to avoid requiring assay adaptation or a complete change of the assay to one with a different measurement basis and thus less or different interferences. In some cases, a combination of methods is needed to increase certainty in the read-outs.

Interferences induced by NMs depend on many different parameters including the type and concentration of NMs, their sterility (lack of endotoxins), the exposure time, the experimental procedure, as well as the stability and behaviour of NMs under the exposure conditions. For example, NMs may agglomerate or sediment in the exposure medium over time, may change their surface speciation and usually acquire and evolve a biomolecule corona. Moreover, dissolution or cellular uptake of NMs could also affect the level of assay interference from the NMs. For example, cell internalised NMs may agglomerate and alter the forward and/or side scattering in flow cytometric assays, resulting in changes in the detected cell size and complexity or granularity of the cell, respectively. Similarly, conditioning of the cell culture medium in response to cellular uptake of NMs and consequent evolution of the NMs corona composition has been demonstrated [74], as has the potential for NMs shedding of serum corona proteins inside cells leading to disruption of cellular proteostasis [75], but these complex considerations are not yet widely factored into *in vitro* toxicity assays or indeed NMs corona evaluation studies.

In enzyme-based analysis, both the kinetic reaction and the end point reaction assays are useful. However, the selection of a kinetic assay where several measurements are taken over the course of an enzymatic reaction, over an end-point assay may be a precautionary action to overcome NMs interference in specific assays [34,76]. Similarly, the use of increased reagent/fluorophore concentration in the assay may reduce NMs interference to negligible levels, as the likelihood of interaction with NMs may decrease at very high reagent/fluorophore concentrations [34,76], but caution should be taken in order not to saturate detectors and to ensure that the selected concentration does not fall within a non-linear response region of the spectrum.

Another mitigation action is washing of the cells with phosphate buffer saline after treatment with the assay reagents in order to reduce the presence of NMs in the test system, and hence the potential interferences. Fixation with glutaraldehyde or formaldehyde followed by washing has also been found to contribute to removal of NMs and their agglomerates, most probably due to crosslinking with the proteins in the NMs' protein corona. However, it was shown that the efficiency of washing steps depends on the adsorption properties (attachment efficiency) of the NMs with the cell membrane, and the route of NMs uptake by the specific cells [34].

Centrifugation has also been proposed as an additional step in toxicity protocols to eliminate residual NMs and reduce interference with readings [77]. However, the efficiency of the centrifugation process to remove NMs may be material specific, dependent on the NMs density and the medium viscosity for example, and is not efficient for all NMs [34,35]. Centrifugation of supernatants prior to their analysis and extensive washing during the ELISA procedure are assumed to remove excessive NMs. Therefore, it is unlikely that remaining NMs interfere with the ELISA assay itself. Additionally, it has been recommended to spike samples with and without NMs with the cytokine of interest and to analyse the level of cytokine recovery in order to provide an indication of the potential interference [50], since adsorption of cytokines by NMs is dependent on the nature and concentration of NMs, as well as on the nature and concentration of the cytokines. In such cases, a dose-response spiking study may be required.

Val *et al.* tested several ways to desorb cytokines from NMs using various detergents, but all tested methods were inefficient [52]. Importantly, several studies also suggested that adsorption could be reduced by working at lower NM concentration or by working with serum or other proteins in the test system [35,51–53] which may limit the adsorption of reagent/analyte/product of interest to the NMs by pre-formation of a protein corona. The presence of proteins in the exposure medium also modulates the biological response to NMs, and indeed may modify the NMs agglomeration and surface presentation to the cells. For this reason, testing should be performed under the relevant exposure settings., Cellular assays are conducted in protein rich medium (cell culture medium enriched with foetal bovine serum or with addition of bovine serum albumin to mimic lung lining fluid), while NMs never have bare surfaces and always have an associated biomolecule layer in real biological media (*e.g.*, serum, cells, tissues) [78]. Thus, dispersion protocol, interference checks and assay protocol should be carefully planned and designed under the relevant experimental conditions, and these should be as relevant as possible for *in vivo* situations. In practice however, the experimental conditions utilised between these different phases of testing are different, which may impede proper comparison and reduce the validity of the data obtained. To avoid this, each phase of testing should be harmonised and synchronized considering realistic exposure conditions.

In all cases, tests for adsorption should be fully integrated into NMs toxicity endpoint assessment and used to interpret experimental data. For example, it has been shown that the percentage of bound cytokines depends solely on the NMs concentration [51], but of course will be modulated by the presence of other proteins due to competitive interactions. Correction of experimental data by subtraction of absorbance / fluorescence / scattering values of reactants and products in the presence of the NMs from those in the absence of NMs (via spiking experiments) may be useful in the case of adsorption [51], but is not always straightforward to perform. Indeed, the test for interferences may be set as a worst-case scenario in their design and the conditions of the test for interferences may be different from the treatment scenario set with cells.

It must be also kept in mind that the modifications of protocols may not always be practical or sufficient to eliminate NMs-induced interferences. Usually, a combination of assays with different measurement principles may provide increased confidence in the data, while sometimes, selection of an alternative method with a different measurement principle may be the only solution.

The risk of interference may be also different when working with cells in suspension compared to adherent cells. In adherent cell cultures, NMs could be adsorbed at the cell surface or trapped in the extra-cellular matrix produced by the cells. In the case of cell suspension, it can be difficult to separate the NMs from the cells for analysis because centrifugation tends to pellet both cells and NMs together, although alternative centrifugation techniques have been proposed [79]. This difficulty arises, for example, during apoptosis evaluation when the analysis of both adherent and detached cells is required [80].

Interference controls

In the pre-study phase, several mitigation actions can be employed to overcome a detected interference between the NMs of interest and the selected assay, which includes a modification of the protocol, adjustment of the time of exposure or a change of the assay itself. However, irrespective of the selected assay, the most important consideration is to include proper interference controls in the experimental design to check reliability and reproducibility of the results. Results on safety assessment of NMs should always be accompanied with the quality assurance data on the assay performance. Fig. 2 shows one example of a 96-well-plate

layout for testing of NMs-induced interferences with absorbance / fluorescence / luminescence / scattering readouts due to the physical presence of the NMs or as a result of NMs interaction with initial or activated reagents. Testing should be performed both in the absence and presence of cells. However, the final experimental design has to be fit to the specific assay applied, as well as the plate format, and thus this should be seen as a guidance to the approach rather than a fixed one-size-fits all layout.

In the case of endotoxin controls, it is recommended to include at least two empty tubes in each sample preparation to determine potential background contamination of LPS before and during sample preparation [81]. The empty tubes from different preparations showed a variation up to 50% confirming the need to include these controls every time the experiment is performed. NMs spiking with a fixed concentration of endotoxin has also been suggested as a positive control, by spiking the samples with 10 EU/mL LPS and determining its recovery, to assess potential interference due to incomplete recovery following binding.

The various mitigation measures are summarised in Table 4 and a first version of the RiskGONE decision tree for addressing interferences of NMs with *in vitro* toxicological test methods is presented schematically in Fig. 3. The decision tree summarises the critical checkpoints where interference with colorimetric/fluorometric *in vitro* assays can be expected and what mitigation action may be undertaken.

The decision tree (Fig. 3) and accompanying checklist (Table 4) is provided here to guide researchers working on *in vitro* test methods and models for safety assessment of NMs in order to gain reliable data for regulators involved in the risk governance of NMs. The decision tree is believed to give added value in nanotoxicology and will be implemented in the RiskGONE cloud platform. We note that all of the issues noted in Table 4 are applicable to HCA approaches also, which will suffer from all above mentioned issues (and mitigations) since HCA is mainly based on traditional biochemical assays (antibodies, reactions for ROS, etc) and the read-out is performed using fluorescence (confocal) microscopy. In the case of development of organ-on-a-chip assays, also called micro-physiological systems, which are advanced *in vitro* cell culture models that utilizes physiologically accurate tissue and organ modelling for toxicology and pharmacology studies [83], the considerations noted in Table 4 around protein binding are important, and the potential for blocking of the tubing to prevent loss of NMs due to binding to tubing needs to be explored.

A brief note on label-free methods identified as alternatives to avoid NMs interference

As noted above, the assays identified in Tables 1–3 are limited to those for which NMs interferences have been identified, and thus, as alternatives we provide some brief notes on alternative methods. We note that the absence of a report on NMs interference with a method is not the same as confirmation that there are no interferences – it is more that this has yet to be confirmed and thus the recommendation around interference controls applies to these methods also and the potential for interference must always be explicitly ruled out / discussed in publications and reports on *in vitro* toxicity testing of NMs.

Electrical impedance

Label-free assays are less prone to interference by NMs since many interferences are related to NMs interactions with assay components (see Tables 1–4). A promising label- and interference-free technique to study cytotoxicity relies on the measurement of the electrical impedance of cells cultured in a specifically designed culture plate containing electrodes (E-plate). By applying an alternating electrical current through the electrodes, one can measure how cells modify the impedance and the extent to which the cells impede the current in the absence and presence of the NMs. When cells are growing, they spread and attach onto the bottom of the cell culture vessel and the impedance rises. By contrast, when cells are dying, they detach and the impedance decreases. Hence, impedance provides information on parameters such as cell number, morphology, attachment and viability in real-time [84]. For cells in suspension, impedance-based flow cytometry (IFC) can be used as an endpoint to study the impedance of each cell [22,85]. Impedance measurements have been demonstrated as an interference-free technique for a variety of NMs such as Au-NMs, Ag NMs, TiO_2-NMs, ZnO-NMs, CuO-NMs, ZrO_2 and, cobalt ferrite NMs [34, 86].

The plating efficiency or colony forming efficiency assay

Loss of cell viability is an ultimate sign of cytotoxicity, and it can be measured by the ability of adherent cells to survive and form colonies using the colony forming efficiency assay (CFE) (also called clonogenic or plating efficiency (PE) assay). Colonies can be counted manually or with an automatized colony counter [87]. Being non-colourimetric and non-fluorescent, the CFE/PE method is especially suitable for the assessment of toxicity of a variety of NMs *in vitro* to avoid interference with the readout of the test method as already demonstrated by several studies [87–95]. The CFE assay has been optimized and standardized for

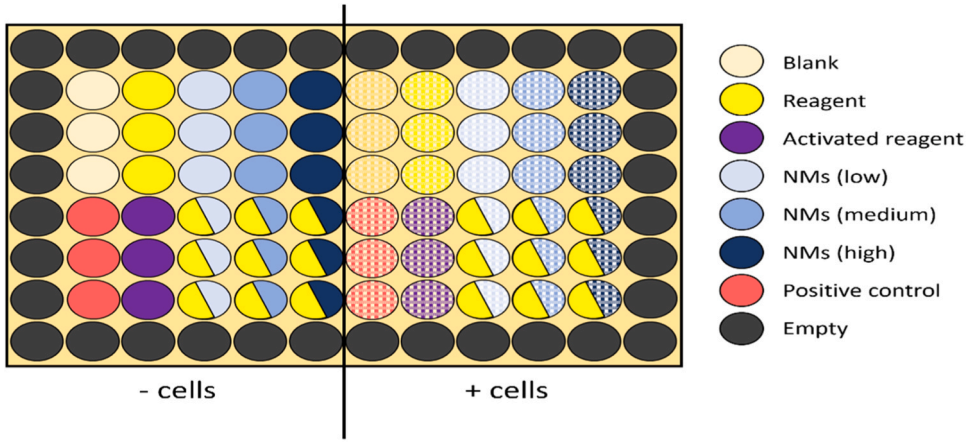

Fig. 2. Example of a plate layout design to assess potential NMs interference. Activated reagent means the reagent after the "reaction" leading to the colour change or signal to be detected. The empty wells are used to prevent evaporation (edge-effects) or can be addressed using newer plate designs with a moat surrounding the edge wells and/or well-designs that allow filling of the complete inter-well space with liquid. The hatching on the wells on the right hand side of the plate indicates the interference controls performed in the presence of cells.

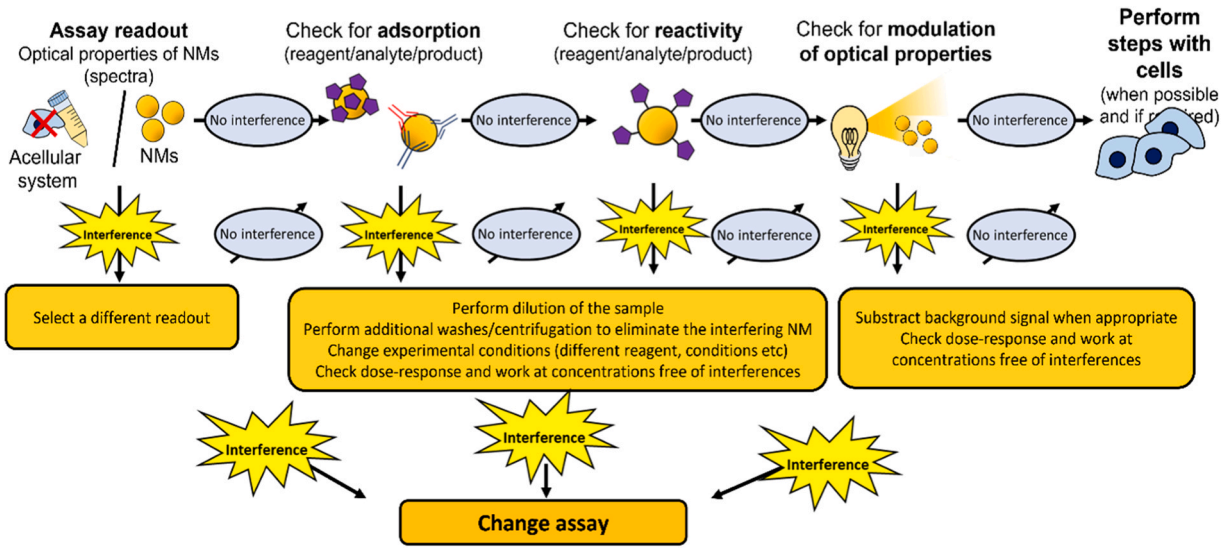

Fig. 3. Decision tree for checking NMs-induced interference with *in vitro* toxicity assay based on colorimetric/fluorometric readouts and suitable mitigation actions.

NMs testing by the JRC's Nanobiosciences Unit and validated in an interlaboratory comparison study for assessing cytotoxicity of NMs by CFE assay [96]. However, this assay requires a longer time 6–10 days (depending on the cell line) compared to other cytotoxicity assays which typically takes 24 or 48 h. On the other hand, longer (more than a week) exposure better mimic a more realistic situation making the assay suitable for determination of lethal concentration for 50% of the cells (LC_{50}). Both cytotoxic and cytostatic (delays or inhibition of the growth of cells, including cancer cells, without killing them**)** effects can be measured using the CFE/PE assay.

Fluorescence lifetime imaging (FLIM)

Fluorescence lifetime imaging (FLIM) is a non-invasive and label-free spectroscopic method that can be utilized to identify oxidative stress in cells and tissues. An auto-fluorescent, endogenous species that is a product of lipid oxidation by ROS, with a characteristic fluorescent lifetime distribution, has been identified as a probe for oxidative stress. Spontaneous Raman spectral analysis at single points of the sample provided molecular vibration information characteristics of the lipid droplets [97]. An example of the application of the method is for the label-free detection and quantification of proteins based on time-gated, wide-field, camera-based UV fluorescence lifetime imaging microscopy to gain lifetime information from each pixel of a sensitive CCD camera. The method relies on differences in the native fluorescence lifetime of proteins and takes advantage of binding-induced lifetime changes for the unequivocal detection and quantification of target proteins [98] Some NMs are inherently efficient two-photon fluorescent indicators including carbon black [99] while a wide range of so-called up-converting NMs have been developed, including fluoride-based inorganic NM such as $NaYF_4$:Er^{3+}, Yb^{3+} [100]. FLIM was used to visualize with high spatial resolution and quantify ultrafine carbon black particles in mouse lung and heart tissues, with results showing that the median numbers of particles in the lung of mice exposed to ultrafine particulate matter of diameter less than 2.5 μm was about twice that of filtered air (FA)-treated mice, and about 1.3 times higher in heart of ultrafine PM-treated mice than in FA-treated mice (Hameed et al., 2022).

Electron spin resonance (ESR) or electron paramagnetic resonance (EPR) spectroscopy

These spectroscopic techniques detect the resonant absorption of microwave radiation by a substance with at least one unpaired electron spin placed in a static magnetic field, and can thus be considered label-free as they use inherent properties of the molecules or NMs. ROS production can be measured in both acellular and cellular systems using ESR/EPR, with the latter also being applicable to cells and whole tissues [44,101,102]. However, the sensitivity of the EPR technique to NM interference has not been systematically assessed [103] and thus users should apply a carefully determined set of interference controls as described above.

Concluding remarks

The specific physicochemical properties of NMs make them prone to interferences with *in vitro* toxicological assays at different levels, which inevitably lead to data misinterpretation. The review of scientific literature presented here provides a comprehensive picture of the potential interferences found in typical NMs-based toxicological assays, focussing on cytotoxicity, oxidative stress, inflammation and protein binding using NMs and the modalities of interference related to NMs properties. NMs, with their higher surface area and strong binding affinities can interfere with *in vitro* toxicity assays in a number of ways, including interfering with the assay readouts as a result of scattering, and by interfering with reagents, analytes or reaction of interest by binding to them and preventing their detection. Interference of NMs with *in vitro* toxicological assays as discussed here is one of the main challenges for hazard characterization and safety assessment of NMs since they can lead to data misinterpretation and discrepancies. As highlighted here, assessment of potential NMs-induced interferences should be included systematically in the experimental design for every type of NMs and every selected toxicity assay to avoid biased results. A catalogue of the NMs that have been shown to display such interferences was compiled in Table S1.

Testing for interferences should be performed at all the identified levels to fully evaluate if and how the results could be affected by the presence on NMs and what types of mitigation actions can be implemented. Indeed, various mitigation strategies have been proposed to overcome the NMs interference challenges identified. If interference cannot be overcome, use of a label-free method is recommended, including some of those presented briefly herein. For each toxicological endpoint, it is highly recommended to run a set of assays relying on different methodologies, to overcome assay interferences, data misinterpretation, and to strengthen the reliability of the results and their acceptance and integration into regulatory decision making.

Finally, a decision tree depicting tests for interferences and suggesting mitigation actions has been developed by the RiskGONE project. This decision tree is being integrated into the RiskGONE cloud platform

(http://www.enaloscloud.novamechanics.com/riskgone.html) as a valuable tool to support scientists and regulators in performing reliable hazard and safety assessments of NMs.

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

No data was used for the research described in the article.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.nantod.2024.102215.

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