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Review

Pitfalls of assays devoted to evaluation of oxidative stress induced by inorganic nanoparticles

Juliana Tournebize [a,1], Anne Sapin-Minet [a], Grzegorz Bartosz [b], Pierre Leroy [a], Ariane Boudier [a,*]

[a] Université de Lorraine, CITHEFOR, EA 3452, Nancy, 54001, France
[b] Department of Molecular Biophysics, University of Lodz, Lodz, Poland

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ABSTRACT

During the last years, there has been a remarkable increase in the use of inorganic nanoparticles (NP) in different applications, including consumer and medical products. Despite these promising applications, the extremely small size of NP allows them to penetrate cells, in which they can interact with intracellular structures causing serious side effects. A number of studies showed that NP cause adverse effects predominantly *via* induction of an oxidative stress – an imbalance between damaging oxidants and protective antioxidants – resulting in inflammation, immune response, cell damages, genotoxicity, etc … Most of the *in vitro* methods used for measurement of oxidative stress biomarkers were designed and standardized for conventional organic, inorganic and biochemical compounds. More recently, these methods have been adapted to studies related to various nanomaterials. Thus, this review is an attempt to highlight some current methods employed in and to provide a critical analysis of the major challenges and issues faced in this emerging field.

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Abbreviations: 8-OH-dG, 8-hydroxy-2-deoxyguanosine; $ABTS^{\bullet+}$, 2,2-azinobis(3-ethyl-benzothiazoline-6-sulfonic acid) radical; CM-H_2DCF-DA, 5-(and-6)-chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate, acetyl ester; CNT, carbon nanotube; DCF, 2′7′-dichlorofluorescein; DMPO, 5,5-dimethyl-1-pyrroline-*N*-oxide; $DPPH^{\bullet}$, 1,1′-diphenyl-2-picrylhydrazyl radical; DTNB, 5,5′-dithio-bis-2-nitrobenzoic acid; Eh, standard redox potential; ELISA, enzyme-linked immunosorbent assay; EMSA, electrophoretic mobility shift assay; ESR, electron spin resonance; FISH, fluorescence *in situ* hybridization; FPG, formamidopyrimidine DNA glycosylase; GM-CSF, granulocyte-macrophage colony-stimulating factor; GSH, reduced glutathione; H_2DCF-DA, 2′7′-dichlorodihydrofluorescein diacetate; IL, interleukin; INT, 2-[4-iodophenyl]-3-[4-nitrophenyl]-5-phenyltetrazolium chloride; LDH, lactate dehydrogenase; LSPR, localized surface plasmon resonance; MDA, malonyldialdehyde; MTT, (4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; MWCNT, multi-walled CNT; NDA, naphthalene-2,3-dicarboxyaldehyde; NF-κB, nuclear factor kappa B; NOX, NADPH oxidase; NP, nanoparticle; OPA, *ortho*-phthaldialdehyde; QD, quantum dot; RNS, reactive nitrogen species; ROS, reactive oxygen species; RT-PCR, reverse transcriptase-polymerase chain reaction; TBA, thiobarbituric assay; TGF-ß, transforming growth factor beta; TNF-α, tumor necrosis factor-alpha; XTT, 2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide; WST-1, 2-(4-iodo-phenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H tetrazolium monosodium salt

* Correspondence to: Université de Lorraine "Cibles thérapeutiques, formulation et expertise préclinique du médicament", Faculty of Pharmacy 5, rue Albert Lebrun, BP 80403, 54001 Nancy Cedex, France. Tel.: +33 3 83 68 23 67; fax: +33 3 83 68 23 01.
E-mail address: ariane.boudier@univ-lorraine.fr (A. Boudier).
[1] Centre for Evaluation and Information on Pharmacodependence-Addictovigilance (CEIP-A) of Nancy, University Hospital of Nancy, Nancy, France.

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1. Introduction

In recent years, plenty of nanomaterials have entered the market of products directly related to human use. For instance, nanoparticles (NP) are frequently used in products which range from cosmetics (TiO_2, Fe_2O_3) to wound dressing (AgNP). Nanoparticles also offer an extraordinary opportunity for applications in medicine [1]. Today, more than 25 nanomedicines have already been approved for human use, such as AmBisome® (nanoformulated Amphotericin B to treat fungi infections) and Feridex IV® (FeO NP as a magnetic imaging contrast agent). These applications were mentioned in recent papers and will not be discussed in this review [2,3]. These NP usually carry out the drug, offering drug protection and/or the ability of drug sustained release. Advanced FDA-approved use of NP includes also quantum dots (QD) in living cell imaging and zirconium oxide (ZrO_2) in bone replacement and prosthetic devices.

Based on their composition, NP can be classified into the following categories according to their nature: organic NP (polymers, dendrimers …) and inorganic ones (Ag, Au, Fe, Pt, TiO_2, QD, silica NP, carbon nanotubes (CNT) …) [3]. Nanoparticle surface can be modified by multiple ligands such as target molecules, polyethylene glycol, thiols, leading to the increase of NP stability and the decrease of their surface reactivity toward biomolecules and cell components [4](Fig. 1A).

The analysis of the numerous studies related to NP in the literature shows that biological effects of NP receive greater and greater attention. Among them, there are several recent reports in which oxidative stress has been identified as an important mechanism for toxicity of all types of NP and especially inorganic ones [5–7]. Furthermore, the evaluation of oxidative stress biomarkers may be a useful way to identify which physicochemical properties of NP are associated with the biological insults, to permit a targeted screening and to allow scientists to generate new and safer NP with this structure-toxicity information in mind.

Oxidative stress describes various deleterious processes resulting from an imbalance between protective antioxidants and damaging oxidants, reactive oxygen and nitrogen species (ROS and RNS, respectively). High levels of ROS or RNS are deleterious to all classes of cell components: lipids, proteins, nucleic acids and other macromolecules (Fig. 1B). However, these species are also

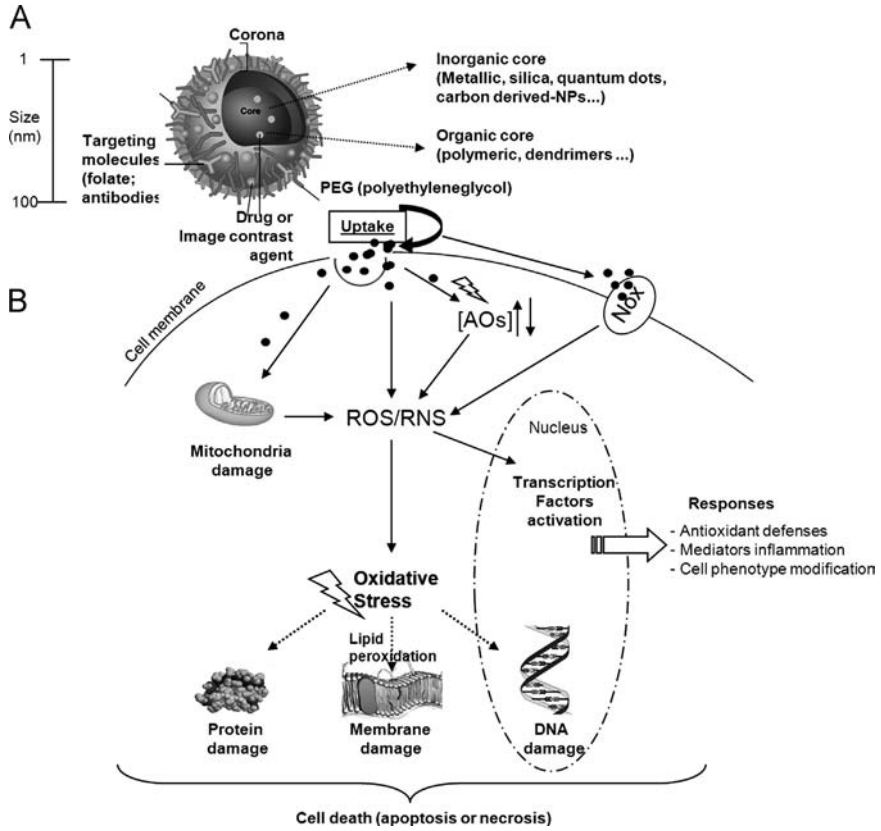

Fig. 1. Interaction of NP with cells: (A) schematic representation of NP types and structures; (B) interaction of NP with cell constituents: interaction with mitochondria, generation of ROS/RNS by NP due to their surface reactivity, interaction with antioxidants with NADPH oxidase (NOX) protein family, transcription factor activation and damage to cell components are shown.

formed in physiology, reacting with cellular components, leading to the activation of intracellular signaling pathways, nuclear transcription factors, inducing gene expression and cell responses such as repair, adaptation or transformation: this process is described as redox signaling. Excellent descriptions of the individual molecules involved in this phenomenon and their scavengers can be found in various reviews [8–10] and in a book [11].

Currently, most of the reported methods to evaluate whether NP can induce oxidative stress have been carried out through classical oxidative stress assays. However, these techniques designed for testing established drugs and chemicals are not always well-adapted to evaluate NP impact. Indeed, some NP can interfere with assay components and readout systems, leading to conflicting reports and the generation of unreliable data, due to their unusual physicochemical properties, including:

- High surface area, and as a consequence increased reactivity;
- Different optical properties that can interfere with fluorescence or visible light absorption detection systems;
- Increased catalytic activity due to enhanced surface energy;
- Magnetic properties that make them redox active and lead to interference with methods based on redox reactions [7,12–21].

Among the different types of NP, we focused on inorganic NP which, due to their high surface reactivity, are more likely to present interferences with classical oxidative stress methods, inducing confusing results. Thus, on the basis of literature, the potentially most relevant mechanisms of inorganic NP for the protection or the induction of oxidative stress *in vitro* are presently reviewed. Then, this review will explain the *in vitro* methods used to evaluate these interactions, and in a last part, in which way NP may interfere with classic oxidative stress assays.

2. How nanoparticles induce and/or prevent oxidative stress

The following section provides a brief overview of the possible cell targets of NP and explains the ability of some inorganic NP – due their surface reactivity – to decrease or increase levels of ROS/RNS.

2.1. Interaction of nanoparticles with cells: target structures

As shown in Fig. 1B, NP may interact in a more or less direct pathway with different cell organelles and species, including: plasma membrane, mitochondria, endoplasmic reticulum, antioxidant compounds, proteins and nucleus. These interactions can lead to changes in cellular redox potential and, as a result, to benefit or adverse effects [22].

First, NP interferes with plasma membrane in different manners. On the one hand, transmembrane proteins, such as NADPH oxidase (NOX), can be directly activated by NP, thus inducing intracellular signaling pathways [23–25]. On the other hand, NP can also be internalized by different mechanisms easing their interaction with internal cell structures. The possible pathways, depending on the physicochemical properties of NP and the exposed cell type (phagocytes *versus* other cell types), have already been reviewed [26] with an extensive overview of the involved mechanisms, and they will not be presently discussed.

Second, once the NP are internalized into the cell, they may interact with organelles, *e.g.* mitochondria (source of superoxide anion ($O_2^{\bullet -}$), hydrogen peroxide (H_2O_2), Ca^{2+}) and endoplasmic reticulum (source of Ca^{2+}), which can lead to further ROS and RNS production. Thus, presence of NP (for example TiO_2, carbon black, fullerol) inside mitochondria of exposed cells induced consequent damages through enhanced generation of ROS [5]. Then, due to their high surface reactivity, NP can interact with cell components such as antioxidants (especially reduced glutathione, GSH, the most abundant antioxidant inside cells). Thus, AuNP were shown to directly react with GSH [6], resulting in deregulation of redox balance and overproduction of ROS/RNS.

As a result, depending on the level of ROS/RNS induced by NP outside or inside the cells, NP can lead to the activation of intracellular signaling pathways (*e.g.* Mitogen-Activated Protein Kinases, MAPK), nuclear transcription factors (*e.g.* activator protein 1 (AP-1)), nuclear factor kappa B (NF-κB), and nuclear factor (erythroid-derived 2-like 2 (Nrf2)) and they can alter gene expression. The induction of adaptive responses including release of inflammation mediators, activation of antioxidant defenses and cell phenotype modification may be expected as a consequence [22]. In some cases, the oxidative stress resulting from an excess of ROS/RNS may overwhelm the antioxidant capacities of cells and may lead to injuries *e.g.* lipid peroxidation, oxidation of proteins and DNA damages, resulting in cell death by apoptosis or necrosis.

2.2. Surface reactivity of nanoparticles

Due their high surface reactivity, some NP (*e.g.* fullerenes, CeO_2, AuNP) could act as scavengers and/or generators of ROS/RNS in cell-free conditions and/or in living organisms [27,28]. As shown in Fig. 2, the reactivity of NP with ROS and RNS species depends on: (i) the catalytic potency of NP surface; (ii) partial dissolution of

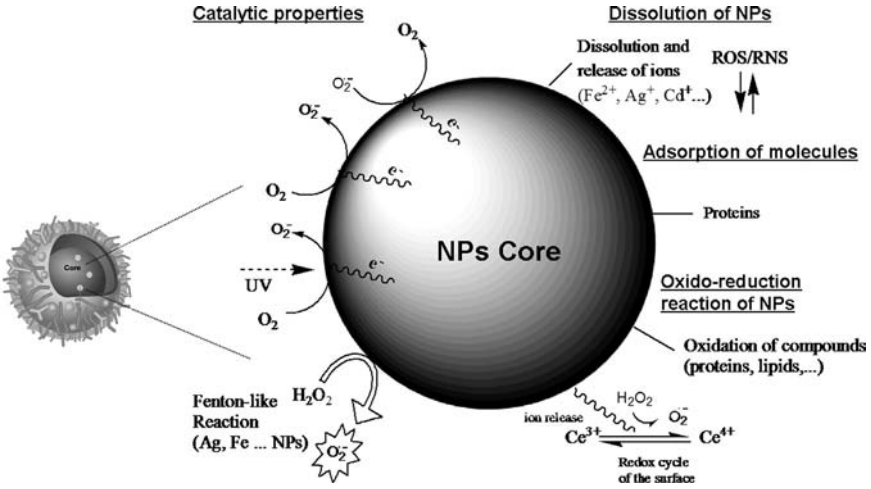

Fig. 2. Surface reactivity of NP: electronic and/or ionic transfer occurring during catalytic, dissolution and redox reactions of NP.

NP and resulting release of metallic ions and (iii) adsorption of molecules onto their surface.

2.2.1. Nanoparticles as radical scavengers

Free radicals (hydroxyl radical ($^{\bullet}OH$) and/or superoxide anion ($O_2^{\bullet -}$)) can be scavenged by NP *via* catalytic reaction on their surface. This was reviewed for CeO_2 NP as well as fullerene and its derivatives [28]. Other NP presented this property such as AgNP coated with aqueous extract of orange peel [29] or stabilized by chitosan [30,31] dendrimers [32], bimetallic NP consisting of Au and Pt [33] metal oxide NP, such as CeO_2, Al_2O_3, Y_2O_3 [34], and also CNT [35].

Nanoparticles themselves can also act as antioxidant delivery systems improving the scavenging properties of the antioxidant alone. For instance, metallic and metal oxide NP has been studied as potential antioxidant delivery systems. Indeed, AuNP enhanced the antioxidant activity of Trolox® (a soluble derivative of vitamin E) [36] and FeO NP encapsulating antioxidant enzymes (superoxide dismutase and catalase) protected cells against H_2O_2-induced death [37].

2.2.2. Nanoparticles as radical generators

Redox reactions following NP dissolution and the resulting release of ions can catalyze Fenton-type reactions. In this context, two kinds of metallic NP can be distinguished: either fully or partially oxidized NP with a standard redox potential (Eh) higher than the Eh of most biological redox couples (including CeO_2, Mn_3O_4 and Co_3O_4 NP [34,38,39]) or fully or partially reduced NP characterized by an Eh lower than the Eh of most biological redox couples, (including Fe°- and the Fe_2O_3-based NP [40]). As shown in Fig. 3, NP with Eh close to Eh of biological redox couples remain chemically stable, whereas NP with higher or lower Eh are usually able to be oxidized, reduced or dissolved, and may induce cell damages.

NPs can generate free radicals or catalyze the formation of free radicals. For example, fullerenes catalyze the production of $O_2^{\bullet -}$ and $^{\bullet}OH$ upon photoexcitation with visible light [41]. Furthermore, AuNP act as catalysts, causing the production of $^{\bullet}NO$ from endogenous *S*-nitrosothiols in blood which is ascribed to the formation of the corresponding covalent Au-thiolate on the surface of AuNP [27,42].

3. Strategies to investigate oxidative stress: interference of nanoparticles

There is a large number of commercial kits used to evaluate whether molecular drugs and chemicals can induce oxidative stress. Today, the same methods have been applied to evaluate the action of NP. However, in many instances these assays were not suitable for NP investigation without prior adaptation, since NP can generate false positive or negative results [7,13,16,17,43] through interferences (Table 1).

3.1. Generalities on interferences generated by NP

In the literature, results obtained with NP in such assays are sometimes associated with bias due to interferences with reagents, released cellular markers or detection systems (Fig. 4). These features will be further discussed but generalities on physicochemical phenomena at the origin of these pitfalls are given below.

Inorganic NP offers a great variety of specific and/or unspecific reactions leading to interferences or reactions with reagents as well as products secreted by cells. These phenomena depend on their unique physico-chemical properties in relationship with their surface chemistry: high area, diversity of electronic configuration linked to enhanced surface energy and catalytic activity and/or magnetic properties. Thus, one has to notice that artefacts can be due to adsorption on particle surface or direct interaction with NP. A decrease of surface reactivity is usually associated with the presence of organic ligands at the surface of the inorganic objects [4].

Moreover, the large number of interference problems occurs by optical interactions. Indeed, noble metal NP (AuNP and AgNP) display localized surface plasmon resonance (LSPR) properties at visible wavelengths which are defined as "the collective oscillation of the conducting electrons of metal NP when their frequency matches that of the incident electromagnetic radiations" [44]. Therefore, strong absorbance band or high scattered light intensity are observed and mainly depend on NP size, shape, core/corona composition and suspension medium (interparticle distance, dielectric constant) [44,45]. Thus, for example, AgNP usually exhibit maximum

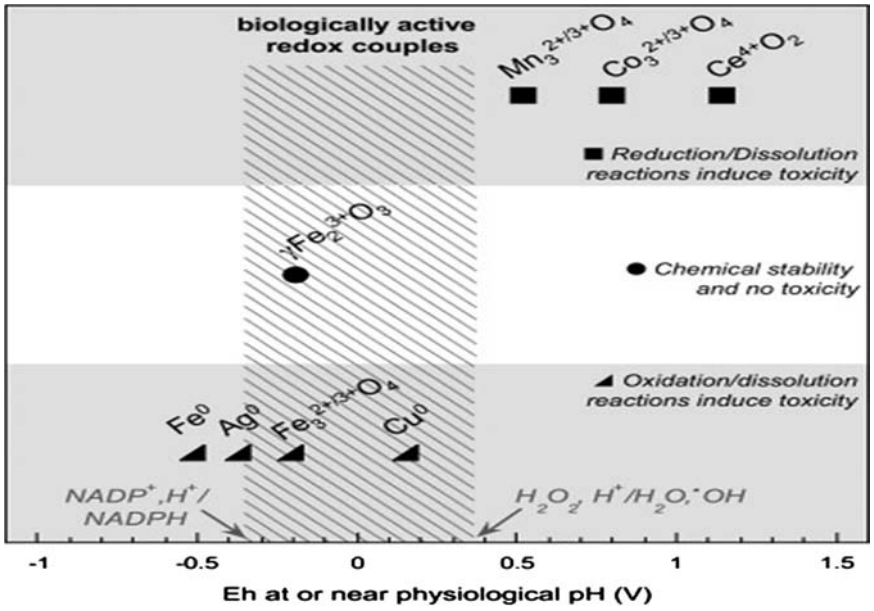

Fig. 3. Plot of the standard potential (Eh) values of various biological redox couples compared to the Eh characteristics of NP. This figure shows the correlation between the ability of metallic nanoparticles to generate a potential toxicity when they are oxidized, reduced or dissolved in biological *in vitro* conditions (reprinted from [31] with permission from Elsevier).

Table 1
Nanoparticle interference with oxidative stress assays.

Methodology	Encountered problem	Proposed solution	NP type / Reference
Cell-free assays			
Spectrophotometric DPPH$^{\bullet}$ assay	Optical interference	Separation of NP from the solution: centrifugation, filtration Use of other detection means: ESR, HPLC	Au/TiO_2 [54], TiO_2 [56] AuNP [36,50], AgNP [29], Diamond NP [50]
Catalytic Reduction of $O_2^{\bullet -}$(produced by xanthine/xanthine oxidase system) by Cytochrome c	Adsorption and inactivation of cytochrome c and xanthine oxidase by NP	Use of other assays to confirm results	MWCNT [35]
TBA assay	Optical interference	Separation of NP from the solution: centrifugation	MWCNT [35], TiO_2 [54]
GSH assay (NDA-GSH adduct)	Fluorescence quenching	Separation of NP from the solution: precipitation followed by a centrifugation step	AuNP [this work]
Cell assays			
DCF assay	Dye adsorption Fluorescence quenching Catalytic activity	Remove NP by washing the NP-treated cells prior to the incubation with probe Restriction of NP concentrations below interfering levels	Dextran-coated FeO [15], Carbon black NP [7,43,77] TiO2, CeO2, AlOOH, Al-Ti-Zr, Ti-Zr, ZrO2, BaSO4, SrCO3 [43]
Colorimetric Griess assay	Optical interference	Restriction of NP concentrations below interfering levels	AuNP [66,78,79]
Ellman assay	Optical interference	Separation of NP by centrifugation from the cell monolayer prior to the incubation with DTNB	$NiFe_2O$ [72], AgNP [80], CuNP [75,81], AuNP [83]
Comet assay	Quenching or enhancement of FI (agglomerates of NP associated with cell DNA)	Restriction of NP concentrations Use of alternative methods to confirm results	Carbon black NP, MWCNT [88] TiO_2 [88,89]
Micronuclei test	Incubation of cytochalasins with NP may decrease the particle uptake and underestimate micronuclei formation	Avoid concomitant incubation of NP and cytochalasins with cells	TiO2 [95]
ELISA	Cytokine adsorption	Adjust culturing (presence or absence of proteins) and exposure conditions (NP and cytokine concentrations)	Carbon black NP [7,12,99,100] TiO2, SiO2 [101]
MTT assay	Optical interference	Development of assays using wavelengths set at 700–800 nm	Single-walled CNT [13,19,102,103], AuNP [this work, 48], TiO_2 [15,49,89]
	MTT-formazan crystals adsorption	Remove NP from the cell monolayer prior to the incubation with tetrazolium salts Use alternative methods (flow cytometry, trypan blue assay, clonogenic assay) Use of water-soluble formazan salts (XTT, WST-1)	[15], AgNP, AgO, CeO_2, CoO and Fe_3O_4 [48]
LDH assay	Metal-catalyzed oxidation of LDH or NADH LDH adsorption Optical interference	Development of assays using wavelengths set at 700–800 nm Remove NP by centrifugation from the cell monolayer	ZnO [7] AuNP [23], PtNP [25] TiO2, AgNP, CuNP [105]

CNT: carbon nanotube; DCF: 2′7′-dichlorodihydrofluorescein; DPPH$^{\bullet}$: 1,1′-diphenyl-2-picrylhydrazyl radical; DTNB: 5,5′-dithio-bis-2-nitrobenzoic acid; ELISA: enzyme-linked immunosorbent assay; ESR: electron spin resonance; GSH: reduced glutathione; LDH: lactate dehydrogenase; MTT: (4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; MWCNT: multi-walled CNT; NDA: naphthalene-2,3-dicarboxyaldehyde; NP: nanoparticle; TBA: thiobarbituric assay; XTT: 2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide; WST-1: 2-(4-iodo-phenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H tetrazolium monosodium salt.

absorbance at 400 nm while spherical AuNP are characterized by a LSPR maximum at 520 nm [45,46]. A wide absorbance range spectrum is also observed for metal oxide NP (AgO, Fe_3O_4, CeO_2, TiO_2 and CoO) [15,47–49]. In addition, molar absorbance of noble metal NP is generally higher than those of many colored reagents used in traditional oxidative stress assays. For example, molar absorbance value for 5 nm diameter citrate-stabilized AuNP ($\varepsilon_{520}=1.2\times10^{+7}\,M^{-1}\,cm^{-1}$) [46] is three orders of magnitude higher than that for 1,1′-diphenyl-2-picrylhydrazyl radical (DPPH$^{\bullet}$) ($\varepsilon_{515}=1.2\times10^{+4}\,M^{-1}\,cm^{-1}$) [50]. Lastly, aggregation of NP is known to increase the optical response: as an example, even though light scattering of 10 nm AuNP may not interfere with read-out systems, this might not be the case with aggregated NP [51]. As a consequence, interferences can be observed in assays using detection systems based on UV–vis spectrum. Lastly, some NP (for example metal noble NP, QD) are characterized by fluorescence properties linked with (LSPR) covering a large range of the UV–visible domain. This may generate again interferences with fluorescent probe and may give false results.

This part of the review is specifically dedicated to identification of the problems due to NP interferences in classical assays that researchers may encounter. In addition, the possible strategies to avoid them are also mentioned.

3.2. Assays to evaluate reactivity between nanoparticles and radicals under cell-free conditions

Numerous chemical assays have been reported to evaluate not only the capacity of antioxidants or NP to scavenge free radicals but also NP ability to generate radicals. The most commonly used methods are based on the monitoring of bleaching rate of colored radicals: 2,2-azinobis(3-ethyl-benzothiazoline-6-sulfonic acid) (ABTS$^{\bullet+}$) and 1,1′-diphenyl-2-picrylhydrazyl radical (DPPH$^{\bullet}$).

3.2.1. Methods to evaluate radical scavenging by nanoparticles

The radical scavenging capacities of NP have been evaluated by incubating them with either colored radicals or $^{\bullet}OH$ or $O_2^{\bullet -}$ previously produced by other sources.

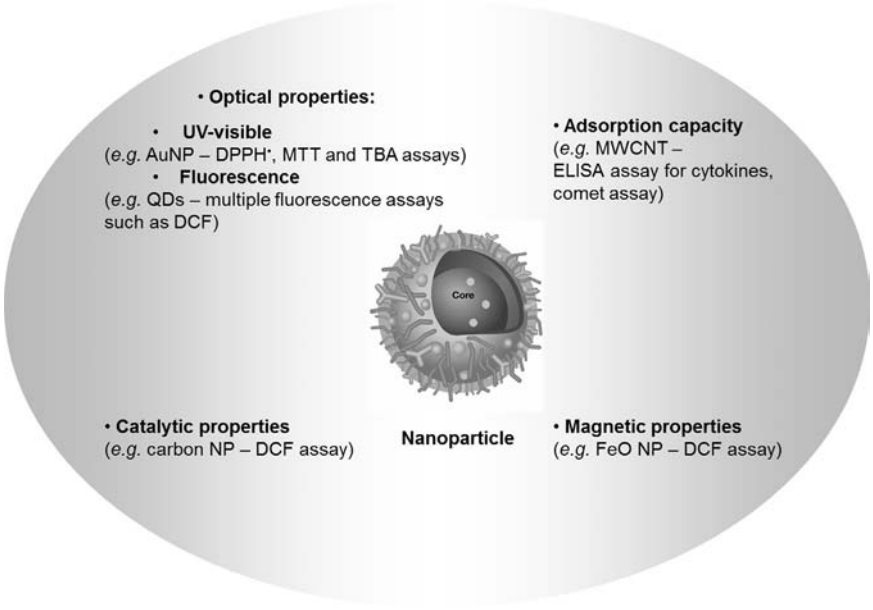

Fig. 4. Nanoparticles specific properties responsible for false-positive and false-negative results with some commonly used oxidative stress assays. *Examples are given as type of NP – assay in which they cause interference. Abbreviations: QD, quantum dots; DPPH•, 1,1-diphenyl-2-picrylhydrazyl radical; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide*; *MWCNT, multiwall carbon nanotubes; DCF, dichlorofluorescein, TBA: thiobarbituric assay.*

As far as colored radicals are concerned, authors usually use either DPPH• [29,36,50–54] or $ABTS^{\bullet+}$ [55]. DPPH• is a stable free radical, while $ABTS^{\bullet+}$ has to be previously generated, *e.g.* by potassium persulfate oxidation [55]. 1,1′-diphenyl-2-picrylhydrazyl radical is very poorly water soluble (need of alcoholic medium); this represents an important limitation when interpreting the role of hydrophilic antioxidants and this may also affect physicochemical integrity of NP. In all cases, the simplest method to estimate antioxidant activity is based on spectrophotometry measurement of the radical color loss (usually followed at 515 nm and 734 nm for DPPH• and $ABTS^{\bullet+}$, respectively) after reduction by the antioxidant. A common problem in the spectrophotometric DPPH• assay relies on the interference caused by optical properties of some NP (Ag and Au) as previously explained. To avoid this problem, various solutions have been suggested either using separation methods to limit spectrum interferences or using other detection means. On the one hand, separation of NP from the solution has been operated by centrifugation [29,54] or filtration [56] before measuring DPPH• absorbance. On the other hand, electron spin resonance (ESR) has been described as an alternative technique to measure the antioxidant properties of AuNP functionalized with Trolox® after reaction with DPPH• [36]. HPLC can also be considered as another alternative approach to determine the antioxidant properties of NP (such as diamond and AuNP). This technique has also been used to further investigate the hypothesized mechanisms occurring during the reaction between NP and the free radical [50].

Other methods have been shown to provide accurate evaluation of NP scavenging radical activity. For example, it has been estimated by monitoring the disappearance of •OH and $O_2^{\bullet-}$ in presence of NP. Hydroxyl radicals can be generated by H_2O_2/UV light process [35] or Fenton reaction [30,32,35] and their consumption by NP can be monitored by spin trapping techniques. In this way, 5,5-dimethyl-1-pyrroline-*N*-oxide (DMPO) probe reacts with •OH to form a stable adduct measured by ESR. A competitive reaction occurs between DMPO and the NP to scavenge •OH. For example, this spin trapping technique was used to evaluate the consumption of $O_2^{\bullet-}$ (produced by xanthine/xanthine oxidase system) by multi-walled CNT (MWCNT) [35]. The authors evaluated MWCNT reactivity with $O_2^{\bullet-}$ by monitoring the reduction of cytochrome c (spectrophotometry at $\lambda = 550$ nm). However, due to high electron affinity and hydrophobic surface, MWCNT were hypothesized to directly react with cytochrome c as well as to adsorb and inactivate both cytochrome c or xanthine oxidase. Thus, to exclude these hypotheses, the authors concluded that other assays were needed [35].

3.2.2. Methods to evaluate radicals generation by nanoparticles

The generation of radicals by NP has been studied by different methods depending of the studied radical type (•OH, $ABTS^{\bullet+}$, $O_2^{\bullet-}$ and •NO). In each of the cases, no interference has been reported.

Firstly, •OH can be generated by the reaction between NP and H_2O_2 and monitored by spin-trapping technique or by deoxyribose degradation with thiobarbituric acid (TBA). Thus, carbon NP and TiO_2 form •OH, which attacks deoxyribose producing malonyldialdehyde (MDA) that, upon heating with TBA at low pH, yielded a pink adduct (absorbance recorded at 532 nm) [35,54]. In these studies, samples were centrifuged to remove NP, avoiding any optical interference. In another work, ABTS assay has also been reported to estimate the generation of •OH by NP [57]. Cerium oxide NP in presence of H_2O_2 and ABTS catalyzed the formation of $ABTS^{\bullet+}$. The production of the radical was measured at 420 nm without CeO_2 NP interference.

Secondly, $O_2^{\bullet-}$ was generated by the reaction between AgNP and H_2O_2. In this study, the radical generation was monitored indirectly, measuring the H_2O_2 concentrations, with Amplex® Red reagent (10-acetyl-3,7-dihydroxyphenoxazine) (reaction with H_2O_2 to produce highly fluorescent resorufin) and directly quantifying $O_2^{\bullet-}$ concentrations by chemiluminescence of a methyl *Cypridina* luciferin analog (2-methyl-6-phenyl-3,7-dihydroimidazo[1,2-a]pyrazin-3-one) [58].

Lastly, reaction between AuNP and •NO donors (for example *S*-nitrosothiols) [27,42] has been used to estimate the generation of the radical nitric oxide (•NO). In this case, a •NO selective carbon fiber electrode was used.

3.2.3. Interaction of nanoparticles with molecules involved in redox homeostasis

Understanding the reactivity between molecules implied in redox homeostasis and NP is crucial to predict NP fate in human body. The reactivity of NP with proteins and reduced glutathione (GSH) has been often studied.

After penetrating the body, NP generally become immediately coated by biological molecules, including proteins. In this way, the

most commonly tested proteins, which are present in human serum, are albumin (the most quantitatively important protein), insulin, γ-globulin and fibrinogen [59–63]. Nanoparticle interactions with these proteins have been evaluated by different assays, without any reported interference, including:

- Protein concentration measurement using bicinchoninic acid assay [59];
- Protein conformational change monitored by one and two dimensional gel electrophoresis [60], fluorescence quenching [61,62] and circular dichroism techniques [62];
- Physicochemical characteristics of NP using dynamic light scattering [61,63], transmission electronic microscopy or isothermal titration calorimetry measurements (enthalpy and entropy analysis) [63].

Nanoparticle interaction with GSH under cell-free conditions has been investigated using fluorogenic reagents, such as rhodamine-based organoselenium probes [6] and *via* determination of NP physicochemical property modifications using, for instance, resonance elastic light scattering – sensitive technique to monitor bioinorganic complexes – and UV–vis spectrophotometry [64,65]. However, the fluorescence intensity of probes can be significantly decreased by the presence of metallic NP (due to optical properties of AuNP for example) giving the false impression of a decrease of GSH content. Thus, our group showed that citrate-stabilized AuNP interfered with the fluorescence emission of naphthalene-2,3-dicarboxyaldehyde (NDA)-GSH adduct (Fig. 5). Since both the excitation and the emission wavelengths of NDA-GSH adduct (472 nm and 528 nm, respectively) lie within the spectrum of visible light, AuNP may absorb not only the emitted NDA-GSH fluorescence but also the excitation energy, thereby preventing excitation of the fluorophore. Thus, potential interferences with NP need to be considered if the investigators wish to reliably use the assays in a quantitative manner. To minimize particle interference, this assay was modified and AuNP were removed by precipitation (acidifying with 0.6 M HCl) followed by a centrifugation step. Fluorescence intensity of the NDA-GSH adducts was then measured and a significant increase of NDA-GSH adduct fluorescence signal (related to GSH concentration) was observed, highlighting AuNP interference with the readout system.

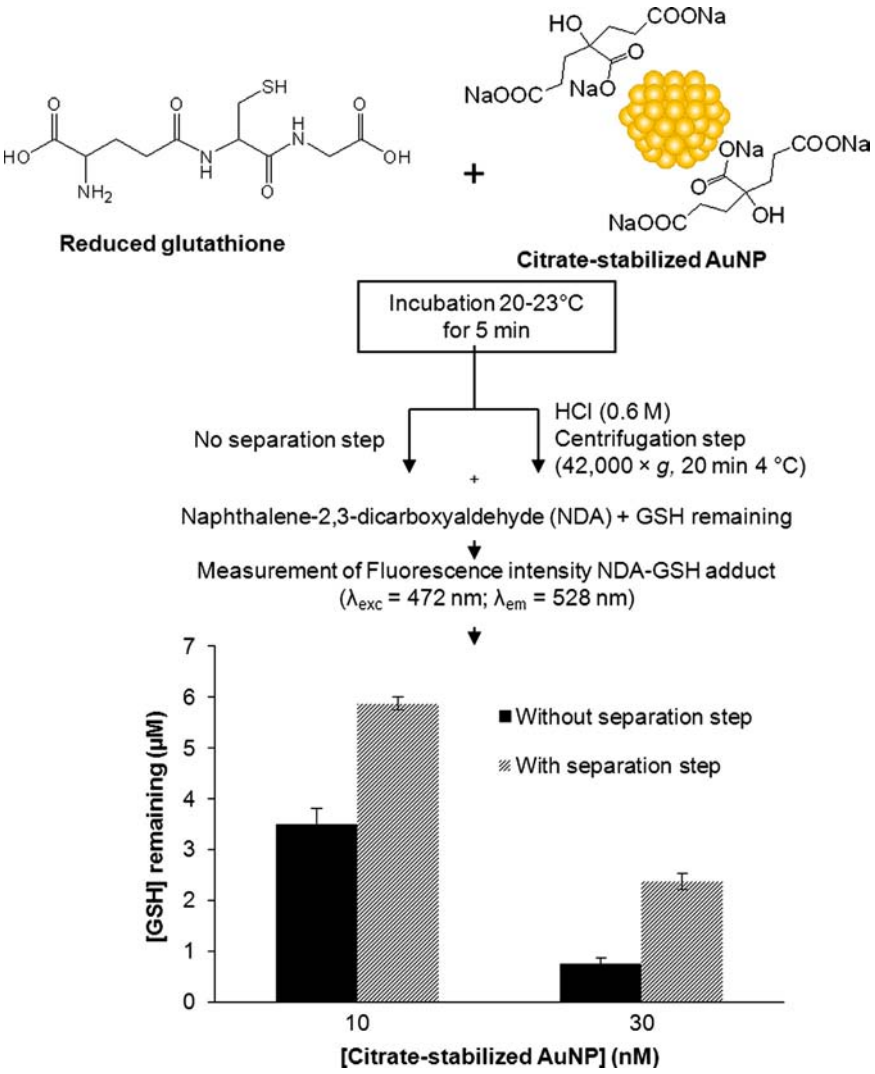

Fig. 5. Interaction in cell-free conditions between reduced glutathione (GSH) (6 μM) and citrate-stabilized AuNP (10 and 30 nM): interference of AuNP in fluorescence read out systems. A solution of citrate-stabilized AuNP (550 μL) at a final concentration of 10 and 30 nm was mixed with 50 μL of GSH (72 μM). Samples were kept at room temperature (20–23 °C) for 5 min. The NP were removed or not by precipitation and 50 μL of HCl (0.6 M) were added to samples. The resulting mixture was centrifuged at 42,000 × g for 20 min at 4 °C. Then, the samples or the supernatant (600 μL) were mixed with 50 μL of NaOH (1 M), 140 μl of borate buffer (1 M, pH 9.2) and 10 μL of NDA (5.4 mM) prepared in ethanol. The mixture was incubated for an additional 10-min period at 4 °C. Fluorescence intensity was read at λ_{exc}=472 nm and λ_{em}=528 nm by using a spectrofluorimeter (Hitachi F-2000, France). Concentrations of GSH were calculated using a calibration curve in the range 1.5–7.5 μM.

3.3. Assays to evaluate the reactivity between NP and cells

The presence of oxidative stress in cells can be evaluated by the direct measurement of radical species, the detection of antioxidant and gene expression levels and the measurement of the resulting damages to cell components. Direct measurement of radical species should be the preferred method, but ROS/RNS are characterized by very short half-lives and remain difficult to directly quantify. For this reason, many scientists prefer to measure the levels of antioxidant enzymes and other redox molecules which act against radical species generated in cells. Several genes that are induced during oxidative stress have also been analyzed. Finally, another approach relies on the evaluation of damages to proteins, DNA, RNA, lipids or other biomolecules. While this is an indirect approach, many markers of damage remain extremely stable and provide, therefore, a more reliable method to measure oxidative stress. The *in vitro* assays used to evaluate these markers are discussed in the following sections.

3.3.1. Methods for measurement of ROS/RNS produced by cells

The induction of oxidative stress by NP in cells can be evaluated by the measurement of ROS and RNS. As far as ROS are concerned, several fluorescent probes are frequently employed, including: 2′ 7′-dichlorodihydrofluorescein diacetate (H_2DCF-DA) [5,54,66–73], 5-(and-6)-chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate, acetyl ester (CM-H_2DCF-DA) [74,75], bis(*p*-methylbenzenesulfonate) dichlorofluorescein [6], hydroethidine [71] and dihydrorhodamine-123 [76]. In all assays, the simplest method relies on the incubation of NP treated cells with the probe, which is then taken up by cultured cells and becomes fluorescent upon intracellular oxidation. Finally, fluorescence intensity is estimated by flow cytometry [54], confocal microscopy [15] or conventional spectrofluorimetry [69].

A consideration, when using fluorescent dyes for quantifying inorganic NP induced oxidative stress, relies on the possibility of their direct interaction with the probe. In this way, various kinds of NP were shown to induce this phenomenon in a concentration- and time-dependent manner. Thus, Doak et al., showed, in a cell-free system, that dextran-coated FeO NP interfered with the fluorescence emission of DCF, depending on the oxidation state of the iron (the interference was more pronounced with Fe_3O_4 than with Fe_2O_3 NP) [15]. They suggested that adsorption could be a reason for the quenching of the fluorescence response. Some studies also noticed fluorescence quenching with carbon black NP while performing DCF assay [7,77]. Carbon black NP can also react directly with H_2DCF-DA due their catalytic activity. This phenomenon has been observed to be incubation-time dependent. In cell-free systems, after 1-h incubation of carbon black NP with H_2DCF-DA, DCF fluorescence was not detectable. However, after 4-h incubation of these NP with the probe, significant intensities of DCF fluorescence were detected following a concentration-dependence manner [7].

To minimize particle interference, the classical DCF assay has been modified: NP suspensions have been removed by washing the NP-treated cells prior to the incubation with DCF [7,43]. In addition, given the fact that the interference of NP is concentration dependent and that all NP cannot be entirely removed from the solution by separation techniques, NP dispersions concentrations should also be limited to values that do not interfere with the measurement of DCF signals.

As far as RNS are concerned, the levels of nitrite ion in culture media are frequently used as an index of $^{\bullet}$NO production. The most frequently applied method to nitrite ion measurement consists in Griess colorimetric technique [66,78,79]. Since nitrite ions are present in supernatant of cells, NP cannot be removed by washing prior to the measurement. Again, limiting NP concentrations to a value that does not interfere with the colorimetric assay is of particular importance to evade false results.

3.3.2. Methods for measurement of antioxidants

Endogenous antioxidants such as GSH are considered to be the first line of cellular defense against radical species. In recent studies, variations of GSH levels after exposure to NP have been investigated using either the colorimetric Ellman's reagent (5,5′-dithio-bis-2-nitrobenzoic acid, DTNB) [72,75,80,81], or fluorogenic reagents (without any reported interference as far as AuNP [6] and silica NP [69] are concerned) such as rhodamine-based organoselenium fluorescent probe [6] and *ortho*-phthaldialdehyde (OPA) [69]. One has to take into consideration that the previously cited NDA and OPA reagents induce the same pathway of GSH adduct formation; but the detection is set in the visible range as far as NDA is concerned and in the UV domain (λ_{exc}=334 nm and λ_{em}=455 nm) as far as OPA is concerned. Therefore, this may imply less interference by endogenous autofluorescence using NDA than OPA [82].

Depletion of GSH has been observed in cells exposed to $NiFe_2O$ NP [72], AgNP [80], CuNP [75,81] and citrate-stabilized AuNP [83]. As previously mentioned in other assays, a separation step by centrifugation was performed to avoid any NP interference.

3.3.3. Methods for measurement of early and late stage of redOx signaling pathway disturbances

The generation of ROS, depletion of antioxidants and decrease of antioxidant enzymes activity correspond to early signals of RedOx signaling pathway disturbance. They remains inevitably linked to an increased risk of oxidative damages to molecules such as lipids and DNA which can finally lead to the induction of an inflammation process but also cell death. These late stages of a RedOx signaling imbalance can be evaluated by using *in vitro* cell assays.

Indeed, lipid peroxidation products, such as malonyldialdehyde (MDA), can be measured by using thiobarbituric acid (TBA) assay [47,75,80], and western blot analysis [84], whereas DNA damages can be assessed by a number of different assays: 8-hydroxy-2-deoxyguanosine (8-OH-dG) detection, comet assay [54,85–87], micronuclei test and fluorescence *in situ* hybridization (FISH) assay [86]. Some studies have reported the interference of inorganic NP such as carbon black, TiO_2 and MWCNT with comet assay [88,89]. Actually, this assay relies on a fluorescent microscopy read-out. Nevertheless, agglomerates of the NP associated with cell DNA clearly induce either quenching or enhancement of fluorescence intensity. As a conclusion, the authors concluded that critical concentrations above which NP massively agglomerate, should not be tested and that alternative methods must be developed to circumvent these problems. The interactions between reactive enzyme of comet assay and inorganic particles may also underestimate the result of comet assay. Kain et al. (2012) described that the decreased activity of the specific DNA repair enzyme formamidopyrimidine DNA glycosylase (FGP) observed with CeO_2 and Co_3O_4 particles was mainly due to FPG-particle interactions [87]. They conclude that measurement of oxidatively damaged DNA in cells exposed to NP may be underestimated in the comet assay due to interactions with this enzyme.

In some studies, micronuclei test have been performed on cells treated with NP. Results showed that fullerene NP did not induce formation of micronuclei in cells [90], which was also the case of Fe_3O_4 [51]. As far as AuNP are concerned, literature data suggests that the effect on micronuclei formation may be not only concentration- but also size-dependent [51,91]. In another study, depending on physico-chemical parameters (crystalline structure, NP size and surface area) tungsten NP incubation on cells was able to cause micronuclei

formation [92]. However, micronuclei test usually depends on the use of cytochalasin B which prevents cell division in order to accumulate multinucleate cells. Molecules belonging to cytochalasin family are described to inhibit actin polymerization and depolymerization [93]. As a result, they are often used as inhibitors of phagocytosis and macropinocytosis (pathways of particle endocytosis) [94]. Therefore, the incubation with this molecule concomitant with NP can affect the particle uptake and underestimate micronuclei formation. This was recently shown with TiO_2 NP [95]. Gonzalez et al. (2011) explored the cellular uptake of amorphous silica NP and their biological effects, depending on presence of serum and the use of cytochalasin-B [96]. Their observations indicated that the experimental design and the choice of the assays are of great importance in nanotoxicology conclusions.

As marker of inflammation and other late consequences of a RedOx imbalance, transcription factors activation (mainly NF-κB pathways) and inflammatory cytokine production are classically monitored. For example, the impact of NP on gene expression of transcription factors has been studied by qualitative methods (electrophoretic mobility shift assay (EMSA)) [70], semiquantitative [66] or quantitative real time reverse transcriptase-polymerase chain reaction (RT-PCR) analysis [84,97,98].

Enzyme-linked immunosorbent assay (ELISA) has also been performed to quantify inflammatory markers in cell culture supernatants after exposure to various inorganic NP including, AgNP [68], AuNP [66], SiO_2 NP [70], carbon black [7,99,100] and QD [97].

Many studies have reported that NP, due to their high adsorption capacity, can directly interact with cytokines released from cells leading to false-negative results. For example, TiO_2 and SiO_2 NP have caused artefacts in proinflammatory interleukin (IL)-6 immunoassay [101] and carbon black particles were found to bind several different cytokines, for example, Granulocyte-Macrophage Colony-Stimulating Factor (GM-CSF), Transforming Growth Factor beta (TGF-ß), Tumor Necrosis Factor-alpha (TNF-α), IL-6 and IL-8 [12,99,100]. Moreover, it was recently reported that the presence of serum proteins could reduce cytokine binding to metal oxide and carbon black NP [7]. Again, the authors advised to limit NP concentrations to a value that does not display cytokine binding to avoid misinterpretation. For instance, they showed that carbon black NP with concentrations up to 50 $\mu g\ cm^{-2}$ do not display cytokine binding in cell-free conditions [7]. However, since cytokine binding is also highly dependent on NP types, cytokine types, and culturing conditions, it is not possible to recommend a general limit for NP concentration, which necessarily must be adapted to the experiment conditions. The NP concentration constitutes also a crucial parameter which could lead to a cytotoxicity induced by oxidative stress. In fact, in the latest consequences, the deep disruption and damages of proteins, lipids, nucleic acids overcome the cell control, repairs and defense mechanisms and lead finally to the cell death.

3.4. *Summary of the strategies used to avoid NP interferences*

As a result, some general adaptations can be summarized to avoid or at least limit NP interferences (Table 1):

- Use well-characterized NP (and not aggregated particles);
- Introduction of a separation step (*e.g.* washing, centrifugation, filtration, NP precipitation) to reduce the amount of NP in presence at the read-out step or restrict NP concentrations below interfering levels, if NP cannot be entirely removed (appropriate controls have to be realized) or use alternative assays not based on spectrophotometry or spectrofluorimetry;
- Use cell suitable exposure conditions and cell-free controls assayed for each concentration of NP and the corresponding signal subtracted from the absorbance of cell groups [47];
- Use suitable NP concentrations on cells below toxicity range.

This last point has to be further developed since interferences have been described in the literature. A classical approach to assess acute cytotoxicity of NP involves the use of tetrazolium salts, such as 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), 2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide (XTT) and 2-(4-iodo-phenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H tetrazolium, monosodium salt (WST-1), that are cleaved in the mitochondria of metabolically active cells to form colored water insoluble (MTT) or soluble (XTT and WST-1) formazan salts [6,13,54,66–70,72,75,81,84]. However, on the one hand, NP may adsorb MTT-formazan salts, limiting their solubilization and consequently causing pitfalls in the estimation of the number of viable cells [13,19,102,103]. (No interference was observed with water-soluble formazan salts, such as WST-1 or XTT [13]). On the other hand, since these assays are based on spectrophotometry detection, NP can generate wavelength-dependent absorbance artefacts. This may lead to the false impression of an improved cytotolerance of NP [17,104]. For example, our group showed that citrate-stabilized AuNP interfered with absorption of MTT assay (detection wavelength usually set between 500 and 600 nm) (Fig. 6A). To confirm this, the data were validated by traditional Trypan Blue exclusion counting assay. In comparison to the last cited test, MTT results appeared to be consistently overestimated ($p < 0.024$) when high concentrations of AuNP were used (>20 nM). Thus, the decreased formation of formazan (due to reduced cell metabolism) was masked by the strong absorbance of AuNP LSPR at 520 nm (Fig. 6B), providing a false

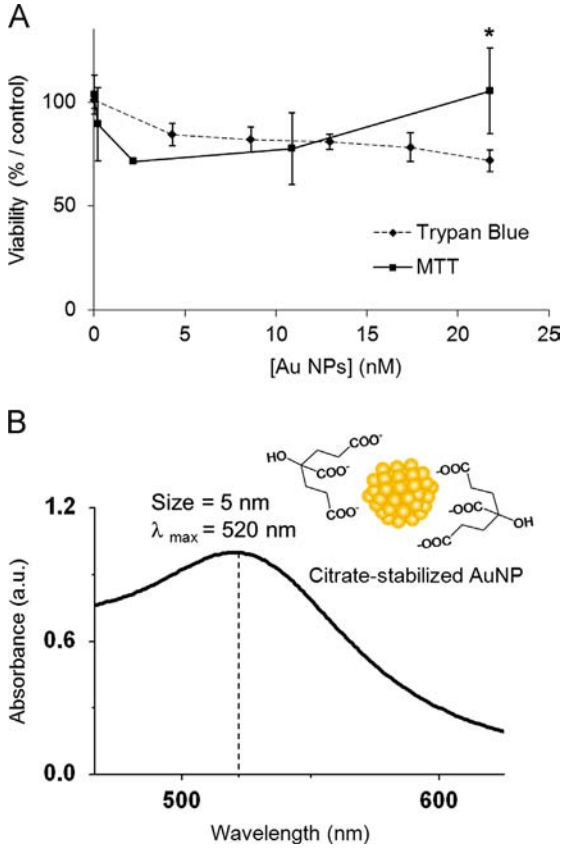

Fig. 6. Interference of citrate-stabilized AuNP with MTT assay: (A) viability studies of rat alveolar macrophages (NR8383) (at 1×10^5 cell/mL) after 24-h exposure with citrate-stabilized AuNP by using MTT assay (■) (96-well plates) (MTT-formazan production measured spectrophotometrically at $\lambda = 540$ nm) and Trypan Blue assay (•) (24-well plates) (observation by optical microscopy). Control cells (unexposed cells with NP) were taken as 100% viability (0% cytotoxicity). Data are expressed as mean and standard error of $n > 3$ replicates in $n > 3$ separate experiments. The non-parametric Mann and Whitney randomised test was performed for statistical calculations. (B) Normalized UV–vis absorption spectra of citrate-stabilized AuNP.

impression of absence of toxicity. The interference with tetrazolium assays is not confined to AuNP alone; it has also been reported for other metallic (AgNP) and metal oxide (AgO, Fe_3O_4, CeO_2, TiO_2 and CoO) NP, which also absorb at the wavelengths used in these tests (from 440 to 600 nm) [15,48,49,88]. As a consequence, the proposed strategies to avoid these interferences rely on:

- Development of assays using wavelengths set at 700–800 nm;
- Remove NP from the cell monolayer prior to the incubation with tetrazolium salts [7];
- Use alternative methods that are not based on optical spectrometry (*e.g.* flow cytometry, Trypan Blue assay, clonogenic assay …) [48,104].

Finally, another common cell viability assay relies on extracellular lactate dehydrogenase (LDH) level released from damaged cells. Lactate dehydrogenase catalyzes the NADH-dependent reversible reaction of lactate oxidation to pyruvate. A second step is often needed: the enzyme diaphorase transfers a proton from $NADH/H^+$ to the yellow tetrazolium salt (2-[4-iodophenyl]-3-[4-nitrophenyl]-5-phenyltetrazolium chloride) (INT), which is reduced to formazan derivative (red) that can be measured by spectrophotometry at 492 nm [7]. The reliability of this assay, when used for measuring NP toxicity, could be influenced by several factors:

- NP (*e.g.* ZnO NP) could cause a decrease in LDH activity by metal-catalyzed oxidation of LDH [7];
- NP (*e.g.* TiO_2, AgNP, CuNP) could be a target on which LDH may be adsorbed [105];
- NP (like AuNP and PtNP) could catalyze NADH oxidation [23,25];
- Presence of NP in supernatant could interfere with read-out signal (usually set in the visible domain, λ=340 or 492 nm if based on NADH [103] or INT absorbance respectively [7])

Again, the previously cited methods (separation step by centrifugation or development of other assay) ought to be performed to avoid any interference.

4. Conclusions

Over the last years, pre-existing oxidative stress methods, currently used to evaluate the potential of xenobiotics to induce an oxidative stress, have also been used to evaluate inorganic NP safety. However, many studies showed that because of the unique properties of these NP, traditional oxidative stress assays should be performed with caution, adapted and sometimes cannot be realized otherwise.

Nanoparticle interference in oxidative stress assays depends on the type of NP (due to the specific NP properties related above) and the suitability of the assay for assessing NP impact should be examined case by case to avoid false results. Some strategies to avoid these interferences when traditional oxidative stress assays are used were described. With this review, we aimed to show how to adapt these methods, highlighting possible artefacts due to nanomaterials in order to obtain reliable measurements of the biological effects of NPs.

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