Toxicology and Applied Pharmacology 299 (2016) 24–29

Contents lists available at ScienceDirect

Toxicology and Applied Pharmacology

journal homepage: www.elsevier.com/locate/ytaap

A redox proteomics approach to investigate the mode of action of nanomaterials

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ARTICLE INFO

Article history:
Received 20 November 2015
Revised 19 January 2016
Accepted 27 January 2016
Available online 28 January 2016

Keywords:
Toxicity mechanisms
Oxidative stress
Acellular tests
Protein carbonylation
Proteomics

ABSTRACT

Numbers of engineered nanomaterials (ENMs) are steadily increasing. Therefore, alternative testing approaches with reduced costs and high predictivity suitable for high throughput screening and prioritization are urgently needed to ensure a fast and effective development of safe products. In parallel, extensive research efforts are targeted to understanding modes of action of ENMs, which may also support the development of new predictive assays. Oxidative stress is a widely accepted paradigm associated with different adverse outcomes of ENMs. It has frequently been identified in *in vitro* and *in vivo* studies and different assays have been developed for this purpose. Fluorescent dye based read-outs are most frequently used for cell testing *in vitro* but may be limited due to possible interference of the ENMs. Recently, other assays have been put forward such as acellular determination of ROS production potential using methods like electron spin resonance, antioxidant quantification or the use of specific sensors. In addition, Omics based approaches have gained increasing attention. In particular, redox proteomics can combine the assessment of oxidative stress with the advantage of getting more detailed mechanistic information. Here we propose a comprehensive testing strategy for assessing the oxidative stress potential of ENMs, which combines acellular methods and fast *in vitro* screening approaches, as well as a more involved detailed redox proteomics approach. This allows for screening and prioritization in a first tier and, if required, also for unraveling mechanistic details down to compromised signaling pathways.

Engineered nanomaterials (ENMs) are nowadays increasingly used in many different consumer products as well as for medical purposes. This poses the question of possible adverse health effects, especially in the long run. Alongside with the continuous discussion on the suitability of current hazard assessment methods (Doak et al., 2012; Hankin et al., 2011; Henkler et al., 2012; Petersen et al., 2015), there are extensive research efforts on new alternative approaches for regulatory testing in particular with an emphasis on high throughput and screening/prioritization methods. In general, *in vitro* methods allow for a higher throughput and for the investigation of the mechanisms of action. Comprehensive systems toxicology approaches are gaining increasing attention in toxicology (Sturla et al., 2014). Systems biology approaches are neither fast nor cheap and thus not suitable for screening purposes. However, they allow for insights into mechanisms of action, which are getting increasingly important in risk assessment, in particular as they may reveal insight into underlying fundamental adverse outcome pathways (AOPs) (Vinken, 2013). AOPs may then improve cross-species comparisons and simplify testing in a sense that the focus can be placed on the most relevant pathways. Thus, mechanistic knowledge of ENMs can enable new regulatory approaches and support read-across and grouping or categorization. In addition, it may be useful for guidance with respect to safe-by-design approaches for ENMs.

Induction of oxidative stress has been reported for many ENMs *in vitro* and *in vivo*, and is probably one of the best understood toxicity principles of ENMs (Nel et al., 2006; Øvrevik et al., 2015). Various adverse outcomes such as inflammation, DNA damage, and general cytotoxicity have been associated with oxidative stress (Halliwell and Whiteman, 2004). Oxidative stress results from an imbalance between reactive oxygen species generation and antioxidant defenses. As a regular byproduct of the respiratory chain and other oxygen consuming reactions reactive oxygen species (ROS) are generated in most compartments of the mammalian cell (Gorlach et al., 2015; Reczek and Chandel, 2015). Oxidative stress through elevated ROS can result from different routes/insults. These include active generation of ROS as a cellular defense mechanism (intracellularly and/or intercellularly)

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http://dx.doi.org/10.1016/j.taap.2016.01.019
0041-008X/

as well as by prooxidant substances such as some ENMs (intracellularly and/or extracellularly). ENMs can be a direct source of ROS through Fenton-type reactions, catalytic chemistry on their surface, and surface reactions driven by excitation of electrons *via* UV-light, or *via* dissolved (metal-) ions (Nel et al., 2006). The characteristics of the ENM primarily determines activity (Tsuruoka et al., 2015). In addition, the environment influences which types of ROS are generated (He et al., 2014). A variety of different methods allow for the detection of ROS and oxidative stress.

1. Acellular assessment of ROS by electron spin resonance and other methods

ROS can be generated by ENMs in fluids in the absence of cells. They can be detected in acellular assays for instance by electron spin resonance (ESR) spectroscopy (He et al., 2014). Detection reagents, called spin traps, form stabilizing adducts with the radical, allowing for the detection of paramagnetic resonance spectra. Three different chemical groups, nitrone, nitroso, and piperidine/pyrrolidine, are used for ESR whereby different spin traps differentiate between the types of radicals. For instance, the cyclic nitrones 5,5,-dimethylpyrroline N-oxide (DMPO) and 5-tertbutoxycarbonyl-5-methyl-1-pyrroline N-oxide (BMPO) are useful for trapping superoxide and hydroxyl radicals while 1-hydroxy-3-carboxy-pyrrolidine (CPH) is useful to probe for singlet oxygen and superoxide radicals (He et al., 2014). Cell-free dispersions of ENMs in water are recommended. They may be diluted using PBS before measurement (Papageorgiou et al., 2007; Shi et al., 2003). More complex cell culture media may also be used. However, in these cases care has to be taken as proteins and antioxidants such as vitamin C may quench the signal (Nymark et al., 2014).

A good correlation between ESR measurements using BMPO and CPH to cellular ROS measurements and cytotoxicity was demonstrated for graphene oxide nanosheets of different oxidation states (Zhang et al., 2015). Particulate matter has been demonstrated to generate hydroxyl radicals in the presence of hydrogen peroxide using DMPO (Shi et al., 2003). A small selection of ENMs has been investigated using DMPO demonstrating a correlation with the potential of generating ROS *in vitro* to inflammation *in vivo* (Rushton et al., 2010). We have recently used DMPO and CPH as complementary spin traps for the investigation of a panel of 24 ENMs, including surface modified variants (Driessen et al., 2015). The combination of the results of two different ESR probes measuring dispersions of ENMs in water yielded a good correlation to the cytotoxicity data (Fig. 1C) (Driessen et al., 2015).

Other acellular methods are also available using DTT as a probe for oxidants (Bates et al., 2015), pH or oxygen sensors (Jensen et al., 2013), vitamin C depletion (Janssen et al., 2015) or oxidant damage in human serum (FRAS assay) (Pal et al., 2014). Especially the latter is a promising approach. FRAS is based on the reduction of a complex of 2,4,6-tripyridyl-striazine (TPTZ) with Fe(III) to a Fe(II)–TPTZ complex with a specific absorbance at 593 nm at pH 3.5 by residual antioxidants present in serum after exposure to ENMs. For the FRAS assay no specialized detection equipment is required, a standard UV–Vis spectrophotometer suffices. It was applied to test 138 ENMs and reliably recognized the highly reactive materials (Hsieh et al., 2013). However, careful improvement of the FRAS protocol allowed to extend the dynamic range towards materials that have less than 1% of the reactivity of the positive benchmarks Mn_2O_3 and CuO. Using the improved FRAS protocol it was possible to differentiate between coated silica materials (W. Wohlleben, personal communication). The materials, which also have been tested in the study by Driessen et al. 2015, ranked SiO_2 naked > SiO_2 amino > SiO_2 phosphate ~ SiO_2 NM203, in accordance with *in vivo* and *in vitro* toxicity (W. Wohlleben, personal communication).

In summary, cell-free methods to determine the oxidative potential of ENMs appear to be a promising tool for a first screening. Our own results suggest a good predictivity of *in vitro* cytotoxicity (Fig. 1) (Driessen et al., 2015). However, some limitations are to be expected when oxidative effects of ENMs shall be predicted for *in vitro* or *in vivo* systems. The local environment of an ENM, *i.e.* cell culture media *in vitro* or body fluids *in vivo*, can influence the reactivity of ENMs in various ways. For instance; they might contain antioxidants, affect ion-release, and alter the ENM surface due to biomolecule corona formation (Monopoli et al., 2012; Nymark et al., 2014). In addition, cellular uptake of ENMs into cells depends often on gravitational settling and other factors. The resulting effective dose likely affects the degree of oxidative stress. In any case, and according to own observations, it is advantageous to combine results from several acellular assays. Specifically for ESR it is recommended to use several spin traps to fully describe ROS generation potential of a given ENM. Acellular assays are comparatively simple and can easily be standardized. However, they need to be combined with cell-based *in vitro* screening methods if effects on biological systems are to be described (Driessen et al., 2015; Zhang et al., 2015).

2. Assessment of ROS *in vitro* using DCF dye based assays

To detect ROS in the cellular environment, a number of fluorescent dye probes are available (Wardman, 2007). The oxidation of the non-fluorescent dichlorodihydrofluorescein ($DCFH_2$) to the fluorescent dichlorofluorescein (DCF) is the most often employed assay. Here, usually $DCFH_2$ diacetate acetyl ester or a derivative thereof is taken up by life cells; esterases hydrolyze the acetate residues which traps the molecule inside the cell where ROS then can oxidize it to the fluorescent DCF.

Recent examples are a size-dependent induction of ROS and upregulation of proteins involved in cellular redox regulation in response to Ag nanoparticles in LoVo cells (Verano-Braga et al., 2014). Similarly, different sizes of Pd and Ni nanoparticles exhibited a size-dependent ROS generation using DCF cell-free as well as *in vitro* on THP-1 cells (Neubauer et al., 2015). Conversely, gold nanoparticles of different sizes showed similar cytotoxicity and ROS profiles (Avalos et al., 2015). Multi walled carbon nanotubes (MWCNTs) more strongly induced ROS in 16HBE14o- cells than onion-like shell-shaped carbon nanoparticles mirroring the effect on viability but not acellular measurements (Kang et al., 2015). Comparing cube, octahedral, and rod shaped CeO_2 nanoparticles, Wang et al. (2015) showed that rod shaped particles induced both lower cytotoxicity and lower ROS generation. Longer single-walled carbon nanotubes were more cytotoxic and also induced stronger ROS generation in NR8383 rat alveolar macrophage cells (Fujita et al., 2015). In human peripheral lymphocytes out of the four nanoparticles of Co_3O_4, Fe_2O_3, SiO_2, Al_2O_3 the latter induced the lowest ROS which was similar to results in cytotoxicity assays (Rajiv et al., 2015). Conversely, MH-S mouse alveolar macrophage cells showed no cytotoxicity and ROS generation in response to Al_2O_3, CeO_2, or SiO_2 nanoparticles, but addition of H_2O_2 revealed concentration-dependent antioxidant properties (Flaherty et al., 2015). We, too, included DCF in the assessment of 24 ENMs and found only MWCNTs to induce dye conversion, contrasting with the results of other methods (Driessen et al., 2015). Similarly, in a study by Decan et al. (2016) acute oxidative stress induced in mouse lung cells following exposure to silica particles measured using DCF was not correlative of later cytotoxicity. These examples show that ROS generation measured by the DCF assay, and consequently cytotoxicity, has been reported for many ENMs. However, correlation between ROS measurement using DCF to cytotoxicity may prove difficult in some cases. The results are cell type and condition-dependent. Similarly, fluorescent dye-based read-outs have also been used for high-content image analysis (Huo et al., 2015; Manshian et al., 2014; Manshian et al., 2015; Wang et al., 2015).

However, there are some limitations to this method for the use in nanotoxicology because of interferences of ENMs (Guadagnini et al., 2015; Hoet et al., 2013; Meng et al., 2009). ENMs may absorb light in relevant parts of the DCF excitation spectrum, exhibit interfering plasmon