Toxicology in Vitro 38 (2017) 136–141

Contents lists available at ScienceDirect

Toxicology in Vitro

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

Impact of cerium oxide nanoparticles shape on their *in vitro* cellular toxicity

Valérie Forest [a,b,c,*], Lara Leclerc [a,b,c], Jean-François Hochepied [d,e], Adeline Trouvé [d,e], Gwendoline Sarry [a,b,c], Jérémie Pourchez [a,b,c]

[a] Ecole Nationale Supérieure des Mines de Saint-Etienne, CIS-EMSE, SAINBIOSE, F-42023 Saint Etienne, France
[b] INSERM, U1059, F-42023 Saint Etienne, France
[c] Université de Lyon, F-69000 Lyon, France
[d] MINES ParisTech, PSL Research University, MAT - Centre des matériaux, CNRS UMR 7633, BP 87, 91003 Evry, France
[e] UCP, ENSTA ParisTech, Université Paris-Saclay, 828 bd des Maréchaux, 91762 Palaiseau Cedex, France

ARTICLE INFO

Article history:
Received 9 August 2016
Received in revised form 15 September 2016
Accepted 26 September 2016
Available online 28 September 2016

Keywords:
Cerium oxide nanoparticles
Nanoceria
Morphology
Toxicity

ABSTRACT

Cerium oxides (CeO_2) nanoparticles, also referred to as nanoceria, are extensively used with a wide range of applications. However, their impact on human health and on the environment is not fully elucidated. The aim of this study was to investigate the influence of the CeO_2 nanoparticles morphology on their *in vitro* toxicity. CeO_2 nanoparticles of similar chemical composition and crystallinity were synthesized, only the shape varied (rods or octahedrons/cubes). Macrophages from the RAW264.7 cell line were exposed to these different samples and the toxicity was evaluated in terms of lactate dehydrogenase (LDH) release, Tumor Necrosis Factor alpha (TNF-α) production and reactive oxygen species (ROS) generation. Results showed no ROS production, whatever the nanoparticle shape. The LDH release and the TNF-α production were significantly and dose-dependently enhanced by rod-like nanoparticles, whereas they did not vary with cubic/octahedral nanoparticles. In conclusion, a strong impact of CeO_2 nanoparticle morphology on their *in vitro* toxicity was clearly demonstrated, underscoring that nanoceria shape should be carefully taken in consideration, especially in a "safer by design" context.

1. Introduction

Cerium belongs to the lanthanide elements also known as rare-earth metals. Cerium oxides (CeO_2) nanoparticles, also referred to as nanoceria, have a wide range of industrial and commercial applications: mainly as fuel additives but also as UV protection (in paints or sunscreens), catalysts, polishing agents, gas sensors, *etc.* (Courbiere et al., 2013; De Marzi et al., 2013; Demokritou et al., 2013; Fisichella et al., 2014; Lin et al., 2006; Lord et al., 2012; Mittal and Pandey, 2014; Peng et al., 2014; Pulido-Reyes et al., 2015; Xia et al., 2008). Recently, they also experienced growing attention for biomedical applications as they were found to exhibit protecting effects against cellular damage induced by toxicants, radiation or in pathological situations such as cardiac or brain ischemia/reperfusion, certain neurological disorders or retinal neurodegeneration (Culcasi et al., 2012; Mittal and Pandey, 2014; Pulido-Reyes et al., 2015). Their potential to behave as anticancer agent has also been explored (Gao et al., 2014; Pulido-Reyes et al., 2015).

Due to this large use, the risk of CeO_2 nanoparticles release in the environment and exposure to humans (especially through inhalation) are potentially growing while their impact on human health and on the ecosystems is still not fully elucidated. This observation has led the Organization for Economic Co-operation and Development (OECD) to classify since 2010 these nanoparticles among the top priority materials for toxicological evaluations (Courbiere et al., 2013; Mittal and Pandey, 2014; OECD Environment, Health and Safety Publications et al., 2010; Peng et al., 2014).

Indeed, the toxicity of nanoceria remains controversial as conflicting results have been reported in the literature. It is generally admitted that cerium oxides have a low toxicity profile (Urner et al., 2014) and it has been shown in different models that although internalized by cells CeO_2 nanoparticles do not trigger inflammation or cytotoxicity (Fisichella et al., 2014; Franchi et al., 2015; Xia et al., 2008). But evidence that cell death can be induced by CeO_2 nanoparticles was also given by Pešić et al. (2015). Similarly, regarding oxidative stress, some studies have reported that cerium oxide nanoparticles can be either pro-oxidative (Pešić et al., 2015) or on the contrary can exhibit anti-oxidant properties (Lord et al., 2012; Mittal and Pandey, 2014; Rosenkranz et al., 2012; Schubert et al., 2006; Xia et al., 2008). Nanoceria can exert a pro-oxidative effect by producing reactive oxygen species (ROS) responsible for cell damages that can themselves potentially lead to cell death, and

* Corresponding author at: École Nationale Supérieure des Mines de Saint-Etienne, 158 cours Fauriel, CS 62362, 42023 Saint-Etienne Cedex 2, France.
E-mail address: vforest@emse.fr (V. Forest).

http://dx.doi.org/10.1016/j.tiv.2016.09.022
0887-2333/

also by inducing changes in the intracellular redox status (Pešić et al., 2015). Thus, some studies have shown that nanoceria can induce oxidative stress either *in vitro* or *in vivo* (Pešić et al., 2015). On the other hand, cerium oxide nanoparticles were found to behave as direct antioxidants by acting as free radical scavengers (especially by interacting with hydroxyl radical [$OH^{\bullet}$], superoxide radical [$O_2^{\bullet -}$] and hydrogen peroxide [H_2O_2]) and therefore protecting cells from death due to oxidative stress (Lord et al., 2012; Mittal and Pandey, 2014; Rosenkranz et al., 2012; Schubert et al., 2006; Xia et al., 2008).

Two main reasons can be evoked to explain these discrepancies. First, no standardized assays exist, making the comparison impossible among the different studies from the literature and therefore making it really difficult to draw firm conclusion on nanoceria toxic potential. Second and most importantly, it seems that numerous parameters are involved in CeO_2 nanoparticle toxicity: factors from the environment but also the intrinsic physico-chemical features of nanoparticles. Regarding the environmental context, pH seems to play a key role as it can drive either the anti-oxidant or pro-oxidant activity of nanoceria (Gao et al., 2014; Lord et al., 2012; Mittal and Pandey, 2014; Pešić et al., 2015; Rosenkranz et al., 2012). Indeed, in a neutral pH environment, CeO_2 nanoparticles are cytoprotective and act as antioxidants whereas at acidic pH they behave as oxidases leading to cytotoxic effects. This might be a reason of different influence of nanoceria on normal and cancer cells as these latter are characterized by an acidic pH environment. This leads to another environmental parameter that could greatly influence the response to nanoceria: the cell type in which biological assays are carried out. This was clearly demonstrated by different studies (Lanone et al., 2009; Pešić et al., 2015; Rosenkranz et al., 2012) where the biological behavior of cells from various cell lines following incubation with CeO_2 nanoparticles were compared. All these studies concluded to a difference of sensitivity between cell types (both between normal cells and cancer cells but also among the different cell lines).

Nanoceria toxicity can also rely on intrinsic nanoparticle physico-chemical characteristics such as surface chemistry, size, shape, dispersion state (Fisichella et al., 2014), the synthesis process (Lord et al., 2012), *etc.*, but more particularly the dual oxidation state of CeO_2 nanoparticles. It is well documented that cerium can exist as Ce^{3+} or Ce^{4+}, this particular configuration allows the unique reduction/oxidation behavior of nanoceria which is responsible for their antioxidant properties (De Marzi et al., 2013; Mittal and Pandey, 2014; Pešić et al., 2015; Pulido-Reyes et al., 2015; Rosenkranz et al., 2012).

Among the different nanoparticle physico-chemical features that could be involved in the nanoceria *in vitro* toxicity, we focused our attention on the influence of the morphology. Therefore the aim of the present study was to investigate if a relationship existed between the cerium oxide nanoparticles shape and the biological response they induced in a model of macrophages in the perspective of designing safer nanoparticles. Several methods are eligible to produce nanoceria with various and controlled shapes, but the point is to change the shape without risking to change other physico-chemical characteristics that could have an impact on toxicity, for instance by using additives that could be difficult to wash thoroughly and could induce toxicity at trace levels, or by choosing very different methods for each shape. So the ideal case consists of selecting a protocol allowing shape tuning just by changing continuously some physico-chemical conditions as temperature, pH or reaction time, keeping the same chemical reactants. After a literature survey, we considered that protocols published by Florea et al. (2013) were the most relevant. Consequently, the obtained nanoparticles were of identical chemical composition and crystallinity, only the morphology varied (rods or octahedrons/cubes). Macrophages from the RAW264.7 cell line were exposed to these different samples and the *in vitro* toxicity was evaluated in terms of lactate dehydrogenase (LDH) release, Tumor Necrosis Factor alpha (TNF-α) production and reactive oxygen species generation.

2. Materials and methods

2.1. Cerium oxide nanoparticle synthesis

The protocols were selected from Florea's article and PhD thesis (Florea et al., 2013). Basically, after mixing at room temperature a solution of Ce(III) salt with the basic solution (soda/ammonia), the precipitate was heated by microwave irradiation at different temperatures and times. Shape tuning (octahedrons, cubes, rods) was expected depending on the conditions. All syntheses were performed with a 10 min heating ramp between room temperature and the desired temperature. The experimental conditions are summarized in Table 1. The given time corresponds to the plateau at the desired temperature. Cooling was relatively fast (a few minutes). In one set of conditions, we compared 2 microwave ovens: Monowave (M) and Synthos (S). After cooling, samples were washed 3 times by centrifugations and redispersed in water.

2.2. Physico-chemical characterization

Size and shape of particles were examined by transmission electron microscopy (TEM) with a TECNAI 20F microscope operating at 200 kV. For cubic or octahedral particles, around 150 particles were measured manually using ImageJ software. Due to their non-spherical shape, it was chosen to measure the biggest dimension. For rod-like particles, diameter and length distributions were measured counting around 150 particles. The mode (most represented class) was also determined in each case.

Nitrogen (N_2) adsorption/desorption isotherms were measured at 77 K using a Micromeritics ASAP 2010 Analyser. After degassing under vacuum at 80 °C, the specific surface area of the powders was determined by applying the Brunauer Emmet Teller (BET) model method on the desorption branch. The equivalent spherical particle diameter was estimated with the following formula $D = 6000/(\rho \times S_{BET})$, where D_{BET} (nm) is the average diameter of the particles, S_{BET} ($m^2 \cdot g^{-1}$) the specific area and ρ ($g \cdot cm^{-3}$) the theoretical density of the powder. For rods, neglecting the tip surface as compared to the lateral surface, we used the formula $d = 4000/(\rho \times S_{BET})$ with d diameter of the rods.

2.3. In vitro toxicity assays

- Cell culture – The RAW 264.7 cell line derived from mice peritoneal macrophages transformed by the Abelson murine leukemia virus and was provided by ATCC Cell Biology Collection (Promochem, LGC, Molsheim, France). Cells were cultured in Dulbecco Modified Eagle Medium (DMEM) complemented with 10% of fetal calf serum and 1% of penicillin-streptomycin (called DMEMc) at 37 °C under a 5% carbon dioxide humidified atmosphere.
- Nanoparticle/cell contacts – Cells were seeded in 96-well-plates (100,000 cells in 50 μL of medium per well) and were allowed to adhere overnight. Nanoparticles were diluted in cell culture medium to reach the following final concentrations: 15, 30, 60 and 120 μg/mL. Nanoparticles were added to cells and further incubated for 24 h.
- Lactate dehydrogenase (LDH) release – To evaluate cell membrane integrity, the cellular release in the supernatant of cytoplasmic lactate dehydrogenase was assessed using the CytoTox-96™ Homogeneous Membrane Integrity Assay (Promega, Charbonnières-les-Bains, France) according to the manufacturer's instructions. The optical density of the samples was determined using a microplate reader (Multiskan RC; Thermolabsystems, Helsinki, Finland) set to 450 nm. Three independent experiments were performed, each in quadruplicate and the activity of the released LDH was reported to that of negative control cells (incubated without nanoparticles). A positive control consisted in the maximal cellular LDH released after cells lysis.
- TNF-α production – After incubation with nanoparticles, the production of TNF-α was assessed in the supernatant using a commercial