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

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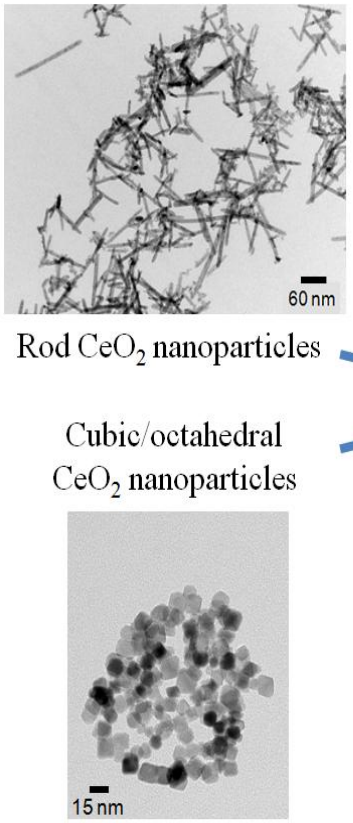
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Graphical abstract



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.

Key-words

Cerium oxide nanoparticles, nanoceria, morphology, toxicity.

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. (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 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 in 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. (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.

Materials and methods

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 minutes 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.

Table 1 – Experimental conditions for the cerium oxide nanoparticle synthesis.

C: concentration, V: volume, T: temperature, t: time, M: Monowave, S: Synthos.

Sample	C(Ce) mol/L	V(Ce) mL	C(NaOH) mol/L	V(NaOH) mL	V(NH3 28%) mL	T(°C)	t(min)	Microwave
CeO_2-R1	$3.8x10^{-3}$	50	12	20	5.4	120	25	M
CeO_2-R2	$3.8x10^{-3}$	50	12	20	5.4	180	45	M
CeO_2-R3	0.1	19	12	20	5.4	180	45	M
CeO_2-O1	0.1	24	/	0	1.4	180	60	M
CeO_2-O2	0.1	19	12	20	5.4	180	45	S

Physico-chemical characterization

Size and shape of particles were examined by transmission electron microscopy (TEM) with a TECNAI 20F microscope operating at 200kV. 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} ($m^2.g^{-1}$) the specific area and ρ ($g.cm^{-3}$) 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.

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 over night. 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 24h.

➢ 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 ELISA Kit (Quantikine® Mouse TNF-α Immunoassay; R&D Systems, Lille, France) according to the manufacturer's instructions. The

optical density of each sample was determined using a microplate reader (Multiskan RC; Thermolabsystems, Helsinki, Finland) set to 450 nm. A standard curve was established and results were expressed in picograms of TNF-α per milliliter of supernatant. Three independent experiments were performed, each in quadruplicate and the production of TNF-α was reported to that of control cells (incubated without nanoparticles).

- Reactive oxygen species (ROS) production – A large array of ROS activity can be assessed with the OxiSelect™ ROS Assay Kit (Euromedex, Mundolsheim, France). The assay uses the conversion of a non-fluorescent substrate, 2.7′-dichlorodihydrofluorescein diacetate that can easily diffuse through cell membranes and be converted into a fluorogenic molecule 2′.7′-dichlorodihydrofluorescein (DCF) in presence of ROS: fluorescence amount is directly related to ROS level. Fluorescence was detected using a Fluoroskan Ascent fluorometer (Ex: 480 nm, Em: 530 nm, Thermolabsystems) after a 90 min incubation of cells with the nanoparticles. A positive control was included incubating cells with H_2O_2 (1 mM). Three independent experiments were performed, each in quadruplicate and the generation of ROS was reported to that of the negative control (cells incubated without nanoparticles).

- Statistical analysis – Analyses and graphics were performed on Prism 6.0c software (GraphPad, San Diego, CA). Significance was established with two-ways ANOVA tests ($p < 0.05$) and Tukey post-tests (***: $P<0.001$ and **: $P<0.01$). Each data point represents the mean of at least three independent experiments and is presented with the arithmetic standard error of the means (± SEM).

Results

Physico-chemical characterization

The physico-chemical features of the different nanoceria used in this study are reported in Table 2.

Table 2 – Physico-chemical features of the different nanoceria.

Nanoparticle	Composition	Particle size (nm) TEM	Shape	Mode (nm)		Specific surface area (m^2/g)	Agglomeration/ aggregation state	Zeta potential pH7 (mV)
				TEM	BET			
CeO_2-R1	CeO_2	5.5(±1.3) x 44(±19)	Rod	4.5-5.5	3.5	151	Low	-26 (± 11)
CeO_2-R2	CeO_2	6.2(±1.1) x 45(±14)	Rod	6.2-7.3	8.5	61	Low	-35 (± 14)
CeO_2-R3	CeO_2	8.1(±1.8) x 70(±34)	Rod	6.8-8.3	14	37	Low	-31 (± 7)
CeO_2-O1	CeO_2	11.3 (±2.8)	Octahedron	10.0-12.0	13	60	Low	-17 (± 5)
CeO_2-O2	CeO_2	16.2 (±3.6)	Cube/octahedron	12.6-15.0	13	61	Low	+9 (± 6)

As illustrated by Figure 1, the different morphologies of the nanoceria could be clearly distinguished using transmission electron microscopy. Samples CeO_2-R1, CeO_2-R2 and CeO_2-R3 are nanorods with increasing dimensions. CeO_2-R1 are thin and short rods, with diameters close to 5 nm and lengths close to 40 nm. CeO_2-R2 samples have more or less the same length as CeO_2-R1, but are slightly thicker rods with diameters around 6 nm, and finally CeO_2-R3 are the biggest rods with diameters close to 8 nm and much longer and polydisperse, around 70 nm. BET equivalent diameters exhibit a much sharper variation than expected by TEM. This may be due to size and shape polydispersity, and the fact that rods could be more or less flat, rather than cylindrical. According to synthesis protocols, CeO_2-R3 was not expected to be rod-like but cubic, in fact the same protocol performed with the Synthos apparatus instead of the monowave produced isotropic particles (CeO_2-O2), but not purely cubic ones, rather a mixture of cubes, octahedrons and cuboctahedrons. This difference

between CeO_2-R3 and CeO_2-O2 is probably due to the sensitiveness of the system to temperature and a better temperature probe for the Synthos, but this discussion is beyond the scope of the article. So at the end, we have 2 families, rods and isotropic particles that can be compared. Interestingly, CeO_2-R2 and CeO_2-O1/O2 exhibit the same specific surface area, but a very different shape, which is very useful to establish if effects are only due to shape. We also observed that agglomeration seems rather weak for all samples. As far as zeta potential in water at pH 7 is concerned, values obtained for anisotropic particles are close to -30 mV but with a high standard deviation (especially for CeO_2-R1 and CeO_2-R2) whereas the zeta potential of isotropic particles is less negative for CeO_2-O1 and slightly positive for CeO_2-O2.

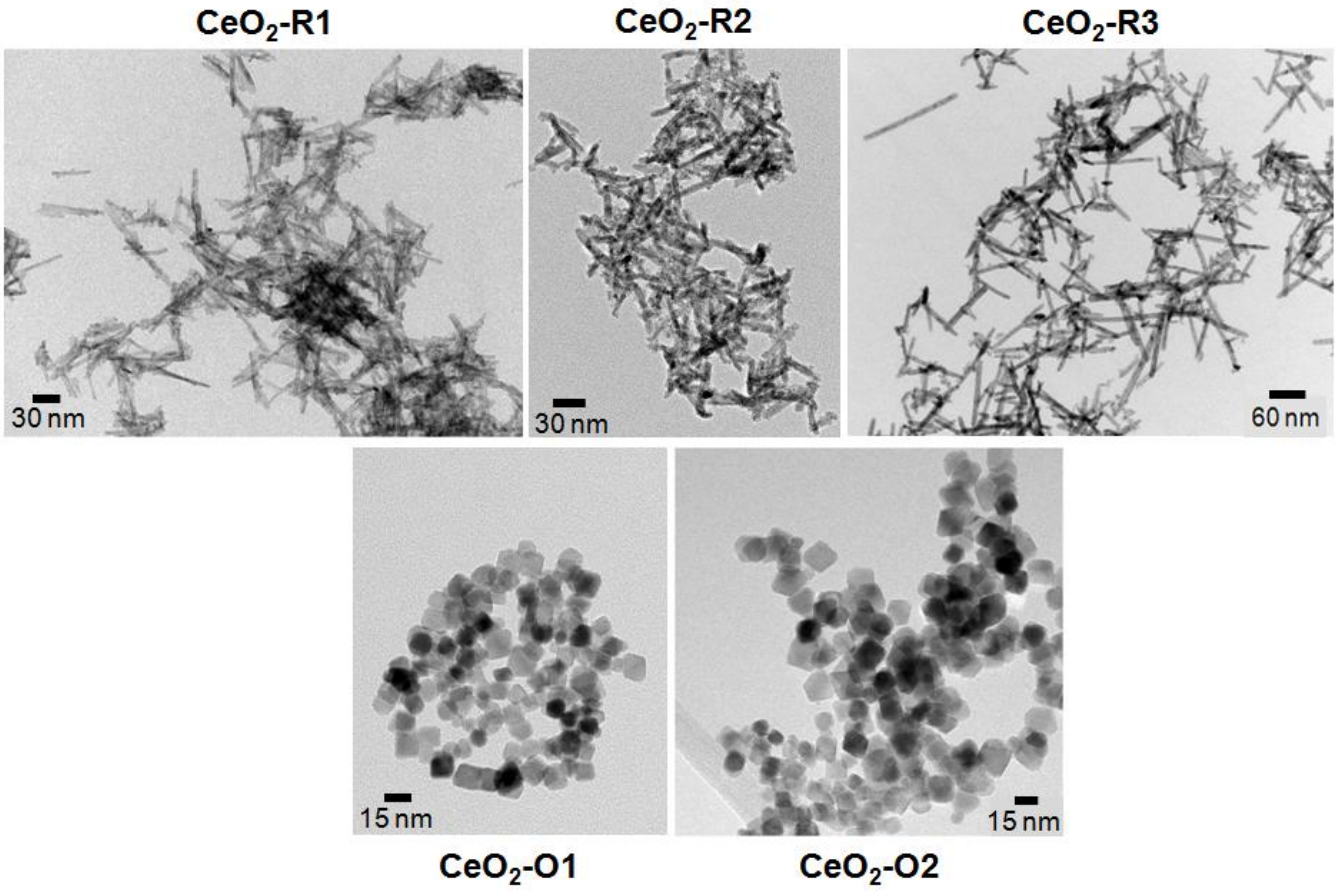


Figure 1 – Transmission electron microscope images of the different nanoceria particles (R = Rods, O = Octahedrons).

In vitro toxicity assays

Figure 2 reports the loss of cell membrane integrity as evaluated by the LDH released from cells after incubation with the different CeO_2 nanoparticles. It clearly appeared that rod particles induced a significant and dose-dependent LDH release whereas that triggered by octahedron particles did not differ from that observed for control cells.

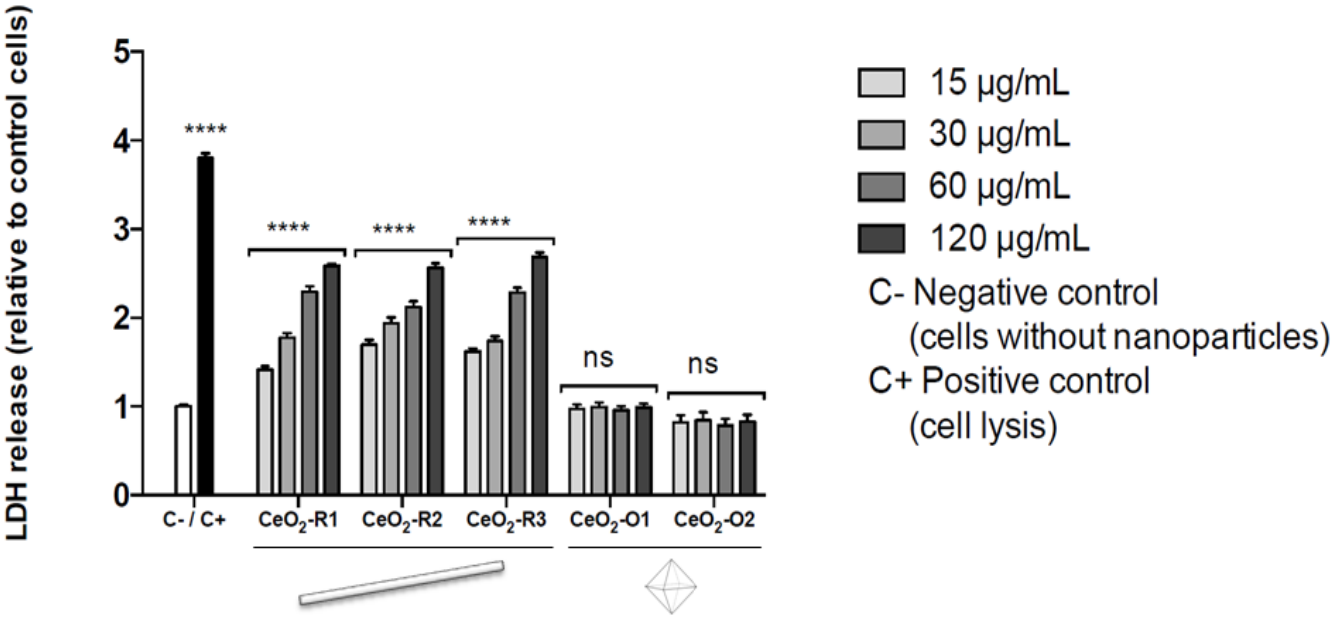


Figure 2 – Loss of cell membrane integrity assessed by LDH released in the cell culture supernatant after incubation with the different types of nanoceria. Results are expressed relative to control cells (incubated without nanoparticles). **** $p<0.0001$ as determined by the Tukey post-test.

As illustrated by Figure 3, the production of TNF-α was significantly and dose-dependently enhanced by rod nanoparticles compared to control cells while the other shape of nanoceria did not induce a significant variation in the expression of this pro-inflammatory cytokine.

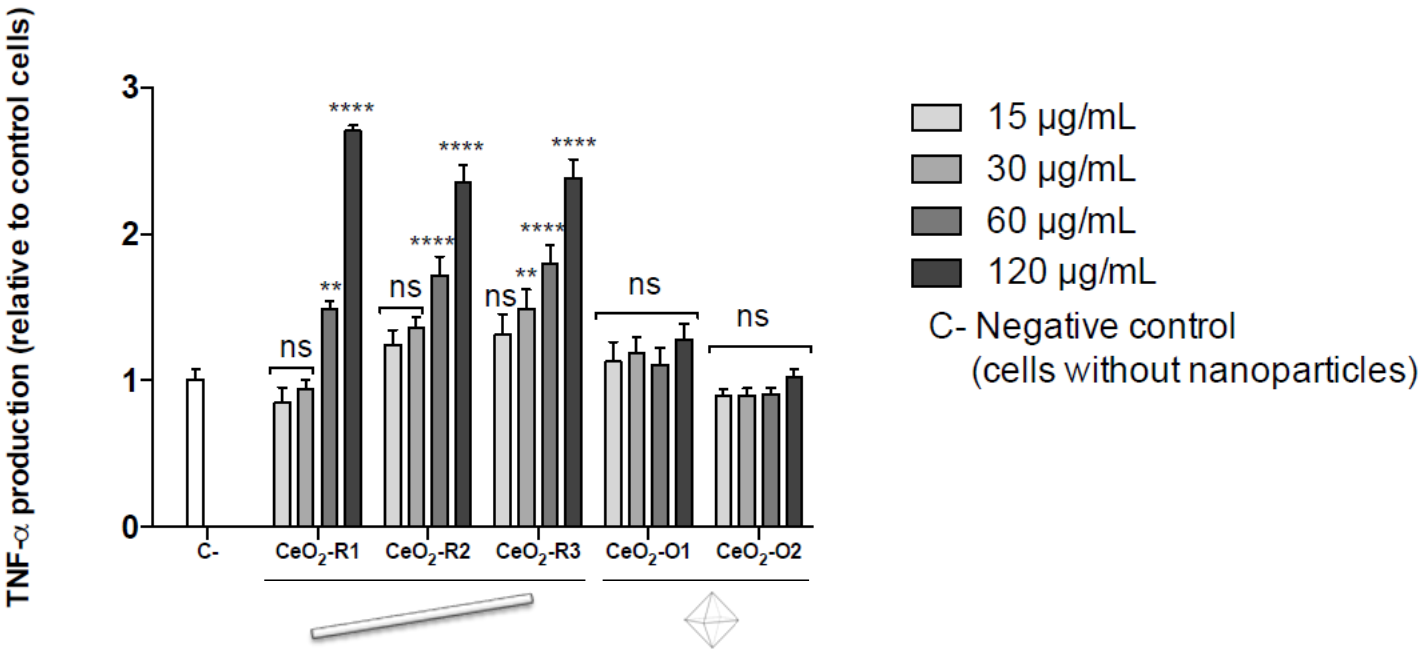


Figure 3 – TNF-α production measured after a 24h incubation with different nanoceria types. Results are expressed relative to control cells (incubated without nanoparticles). ** $p<0.01$ and **** $p<0.0001$ as determined by the Tukey post-test.

Oxidative stress was evaluated by the assessment of the amount of ROS produced relative to control cells (incubated without nanoparticles). As shown by Figure 4, no significant ROS production was observed, whatever the type or concentration of nanoparticles.

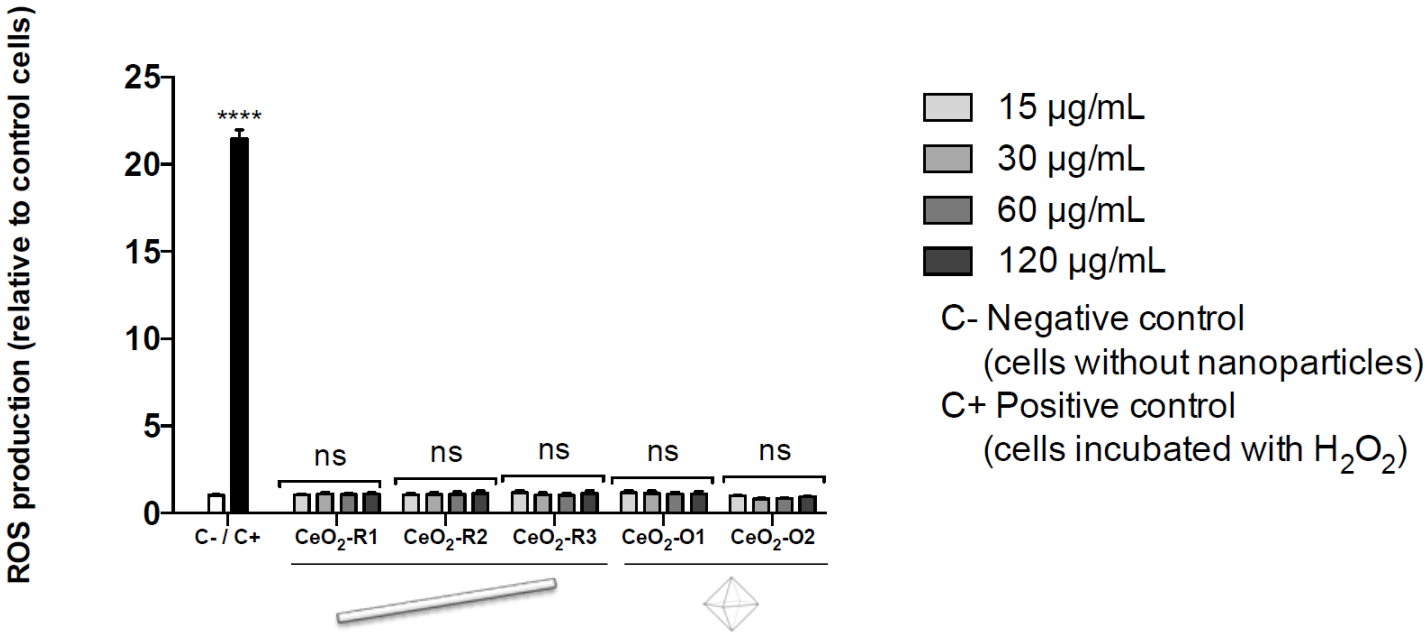


Figure 4 – ROS production assessed 90 min after incubation with the different nanoceria. Results are expressed relative to control cells (incubated without nanoparticles). **** $p<0.0001$ as determined by the Tukey post-test.

Discussion

Nanoceria toxicity remains controversial and data are still missing to firmly conclude on this issue. Among the numerous parameters that could be involved in the nanoCeO_2 toxicity we have chosen to investigate more particularly the impact of the nanoparticle morphology. For that purpose we used a synthesis mode allowing producing different types of particles of well-controlled physicochemical features, varying only in terms of shape (rod versus cube/octahedron). The subsequent study of the biological activity induced in macrophages after incubation with the different nanoceria showed that the extracellular release of LDH (Figure 2) and the production of the pro-inflammatory cytokine TNF-α (Figure 3) exhibited similar profiles. Compared to what was observed in control cells, they were significantly and dose-dependently enhanced by rod-like nanoparticles, whereas they did not vary with

cubic/octahedral nanoparticles. These results clearly argue for a morphological effect of nanoCeO_2. It is also noteworthy to mention that no size effect was observed among the rod nanoparticles as the LDH release and TNF-α levels were similar between CeO_2-R1, CeO_2-R2 and CeO_2-R3 which greatly vary in size (CeO_2-R3 rods are almost two times longer than CeO_2-R1 and CeO_2-R2, Table 2). Also, we did not observe any correlation between the nanoceria zeta potential and their toxicity. Indeed, the LDH release and TNF-α production triggered by CeO_2-O1 and CeO_2-O2 were similar whereas they exhibited a very different zeta potential (-17 and +9 mV respectively). This remark also applies to the rods even if the difference between their zeta potential was lower (Table 2). This strengthens our previous conclusion of the impact of the nanoceria shape (and not their charge) on their toxicity. Finally, we also showed that nanoceria did not induce any ROS production, whatever their morphology (Figure 4).

These results are only partly consistent with the literature. For instance, regarding the production of ROS, as we did, Xia et al. (Xia et al., 2008) and Urner et al. (Urner et al., 2014) reported that nanoceria did not trigger the production of ROS. On the contrary, Mittal et al. (Mittal and Pandey, 2014) and Lin et al. (Lin et al., 2006) found that nanoCeO_2 were able to induce a significant oxidative stress. Another discrepancy with these same studies regards the cytotoxicty induced. Indeed, Mittal et al. and Lin et al. (Lin et al., 2006; Mittal and Pandey, 2014), observed that nanoceria reduced cell viability, whereas Xia et al. considered it as non toxic. We were in a more nuanced position as we reported that toxicity depends on the morphology as it was significantly enhanced by rod-like nanoparticles but not by cube or octahedron-like nanoparticles. But even more interesting is the comparison with results from Wang et al. paper (Wang et al., 2015) which study design was quite similar to ours. Although they used another cell type (human hepatocellular carcinoma cells, HepG2), they compared the toxicity of three kinds of nanoCeO_2 with different morphologies (cube-, octahedron-, and

rod-like), using similar ranges of nanoceria concentrations as we did. However, unlike us, they showed that all three types of nanoparticles caused cytotoxicity. They further reported that the toxicity of cube-like nanoCeO_2 was the highest, that of the rod-like was the lowest and that of the octahedron-like was intermediate. They linked this toxicity level to the specific surface area as they observed that a smaller surface area was correlated with a higher cytotoxicity.

The comparison of these different studies illustrates how difficult it is to reach firm conclusions on the nanoceria toxicity. This could be partly explained by the fact that the biological response often depends on the cellular context, especially the pH (Franchi et al., 2015) and the cell type. The cell type-specific response to particle exposure has already been well established in different models. For instance, Lanone et al. (Lanone et al., 2009) evaluated the toxic effect of 24 nanoparticles, including nanoceria, of similar equivalent spherical diameter and various elemental compositions on two human pulmonary cell lines: A549 and THP-1. Results clearly highlighted the difference of sensitivity between cell types. Similarly, Pešić et al. (Pešić et al., 2015) compared the ROS production induced by nanoCeO_2 in human normal and cancer cell lines. Different responses were observed underlining the selectivity towards human cancer cells. In another model, it was also demonstrated that remarkably more ROS were formed in alveolar macrophages compared to alveolar epithelial cells (Urner et al., 2014). Regarding the protective effects of nanoCeO_2, it was demonstrated that they could protect cells from harmful effects of radiation and oxidative stress, although this protection was cell type specific (Mittal and Pandey, 2014). Also, nanoCeO_2 showed no toxic effect on normal breast epithelial cells and only a slight effect on breast cancer cells, but were able to protect the normal epithelial cells from radiation while in contrast no such protection was observed for the breast cancer cells (Rosenkranz et al., 2012).

Another reason explaining why it is so challenging to conclude on nanoceria toxicity is that the underlying mechanisms are particularly complex to investigate. This is especially true for ROS that are unstable and highly reactive species. In this case, the kinetics aspect is of paramount importance and has to be taken into account to determine the optimal detection window. Thus, it has been reported that nanoCeO_2 were able to produce ROS in a concentration and time dependent manner up to 6 hours (Mittal and Pandey, 2014). But level of ROS was found to be decreased at 24 hours. This may be either due to the increase in amount of cell death or generation of ROS stabilized after a certain time period. Furthermore, a production of ROS can be compensated or masked either by the scavenger activity of nanoCeO_2 or by the cell antioxidant defense system. This is why a complete and thorough study should be undertaken to better understand the oxidative stress mechanism induced by nanoCeO_2. Especially the variation of the antioxidant enzymes level such as the superoxide dismutase (SOD), glutathione (GSH) and catalase should be studied.

It should also be mentioned that care must be taken when concluding on the nanoceria toxicity only on the basis of *in vitro* experiments. Indeed, although many *in vitro* studies have concluded that nanoCeO_2 can be regarded as relatively safe nanoparticles, *in vivo* models allowed underscoring cytotoxicity and inflammation, as well as potential fibrogenicity (Demokritou et al., 2013). For example, it was reported that after oral or intraperitoneal administration of nanoCeO_2 to CD-1 mouse, even if no lethal effect occurred, nanoCeO_2 could induce inflammation status, revealed by cellular, toxicological, and ultrastructural observations (Poma et al., 2014). Similarly, intratracheal instillation of CeO_2 nanoparticles in Sprague-Dawley rats was found to result in liver damage (Nalabotu et al., 2011). Finally, Pulido-Reyes et al. (Pulido-Reyes et al., 2015) studied the effect of five different nanoCeO2 on an aquatic microorganism. They concluded that neither shape, concentration, synthesis

method, surface charge, nor nominal size had any influence in the observed biological activity. Discrepancies between *in vitro* and *in vivo* toxicity evaluations may be attributed to particle transformations in the microenvironments of cell culture media enriched with serum proteins versus the alveolar lining fluid of the animal lung (Demokritou et al., 2013). Nanoparticles suspended in physiological media may experience agglomeration and the formation of a protein corona on the particle surface, and these transformations may have a strong impact on subsequent cell/nanoparticle interactions (Forest et al., 2015). Therefore widely used *in vitro* toxicity assays, such as LDH and MTT assays, may not be predictive of toxicological outcomes observed *in vivo.* Consequently, further investigations are required.

Conclusion

In the present paper, we clearly demonstrated an impact of cerium oxide nanoparticle morphology on their *in vitro* toxicity. Indeed, unlike cubic/octahedral nanoparticles, rod-like nanoparticles significantly and dose-dependently enhanced pro-inflammatory and cytotoxicity responses. Although other complementary studies are needed, this strongly argues for the fact that the shape of the nanoceria is an aspect that should not be neglected, especially in a "safer by design" context.

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