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Internalization and toxicological mechanisms of uncoated and PVP-coated cerium oxide nanoparticles in the freshwater alga Chlamydomonas reinhardtii †

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Due to the wide range of applications of cerium oxide nanoparticles (CeO2NPs), a risk assessment of their biological effects using environmentally relevant species becomes highly important. There are contradictory reports on the effects of CeO2NPs, which may be related to the use of different types of nanoparticles (NPs) and coatings. CeO2NPs may act as an oxidant causing toxicity or as an antioxidant able to scavenge free radicals. As a consequence of such complexity, the toxicological behaviour of these NPs is still poorly understood. Moreover, little is known about the internalization process of CeO2NPs in algae. There is evidence of CeO2NPs-internalization by the green algae Chlamydomonas reinhardtii, but the mechanism and route of uptake are still unknown. In this study, we used an uncoated and different polyvinylpyrrolidone (PVP)-coated CeO2NPs with the aim of identifying their toxicological mechanisms to C. reinhardtii and exploring their possible internalization. Our results showed that PVP-coated CeO2NPs significantly increased the formation of reactive oxygen species in exposed cells, indicating that oxidative stress is an important toxicity mechanism for these particles. Direct contact and damage of the cellular membrane was identified as the mechanism causing the toxicity of uncoated NPs. From experiments with endocytosis inhibitors, clathrin-dependent endocytosis was revealed as the main internalization route for all NPs. However, uncoated CeO2NPs led to severe cellular membrane damage, direct passage of NPs through membrane holes could not be discarded. To our knowledge, this is the first report with evidences of direct linking between NP internalization and a specific endocytic pathway. The results presented here will help to unravel the toxicological mechanism and behaviour of CeO2NPs and provide input information for the Environmental Health and Safety assessment of CeO2NPs.

Introduction

Surface coatings of NPs are applied to selectively change or influence several particle properties1. The surface of a particle can be covered with a wide variety of elements such as dendrimers2, polymers3, metal4, metalloid5, peptides6 or polysaccharides7. The increase of particle stability8, the prevention of particle core dissolution8, the protection of particle functionality or the enhancement of biocompatibility9 are some different purposes to use these coatings. However, since coatings change the surface and physicochemical properties of NPs in comparison with bare NPs, it is essential to completely study the implications of their use, giving that they may have an important role on how coated NPs interact with biological systems and, therefore, on their (eco)toxicological effects.

Cerium oxide nanoparticles (CeO2NPs) have become an exceptionally versatile material due to their high surface area and redox activity10,11. A diversity of applications has emerged due to their singular surface chemistry. Applications include electrochemical bio-sensors12, radiation protector13, corrosion-resistant coatings14 and antioxidant agents in the biomedical field15-17, among others18. Controlling the surface properties of CeO2NPs is essential for them to behave as designed. The reactivity of CeO2NPs strongly depends on NP size. However, it has also been shown that surface coatings play a significant role18. In recent years, CeO2NPs have been synthesized and functionalized with a variety of molecules such as small ligands19, polymers20-22, surfactants23 and other organic molecules24 using different strategies25.
Due to their variety of applications and extensive use, CeO₂NPs may be released to the aquatic environment, where the interaction with aquatic organisms is unavoidable. Until now, several authors showed that CeO₂NPs may cause some harmful effects in different environmentally relevant microorganisms ²⁶⁻²⁹. Considerable efforts have been undertaken in order to understand the toxicological mechanisms of CeO₂NPs ²⁸, ³⁰, which is a difficult task due to the existence of contradictory results ²⁹, ³¹⁻³³. It has been found that CeO₂NPs displays enzyme-mimicking activities ²⁵, ³⁴, ³⁵, protecting against oxidative stress-induced cellular damages, but also they have showed toxic effects to different organisms and cell lines ³⁶⁻³⁸. Moreover, although some authors have shown their skepticism about particle internalization in algal cells ³⁹, it has been recently revealed that internalization processes might be involved in the effects of CeO₂NPs in the model aquatic algae Chlamydomonas reinhardtii (C. reinhardtii) ⁴⁰. However, it is unclear how CeO₂NPs can pass through cell envelopes. Overall, whether CeO₂NPs are internalized in green algae by using some endocytic routes or by using other mechanisms is not fully understood. Further research is also needed on the effect of coatings on toxicity and NP uptake. In this regard, some results point towards an enhanced internalization of nanomaterials with organic coatings ⁴⁰⁻⁴².

Furthermore, the biological effect of coatings on the toxicity of CeO₂NPs to environmentally relevant microorganisms has not been completely assessed. On the one hand, several authors have found that uncoated CeO₂NPs are more toxic than citrate-coated NPs towards a snail ⁴³ and amphibian larvae ⁴⁴. On the other hand, it has been shown that poly(acrylic acid)-stabilised CeO₂NPs were more toxic than pristine forms to model alga Pseudokirchneriella subcapitata ⁴⁵. Either way, whether CeO₂NPs are being synthesized with coatings to enhance their colloidal stability or to develop new functionalities, they thus should be evaluated with those coatings as they will be possibly found in the environment in that form. In this study, we used bare and PVP-coated CeO₂NPs with the aim of identifying the mechanism by which they affect the green algae C. reinhardtii. Attention was also centered on the impact of different coatings and on the potential routes of uptake and internalization.

Materials and Methods

Synthesis of CeO₂NPs

Three PVP capped CeO₂NPs were synthesized using the method described by Briffa et al ⁴². Briefly, 130 mg of Ce(NO₃)₃ were dissolved in a 5 mM solution of PVP, with different molecular weights (10, 40 and 360 kDa). The mixture was heated for 3 h at 105 °C, after which the reaction was quenched and excess PVP was removed using acetone. Centrifuging at 4000 rpm at room temperature for 10 minutes resulted in a yellow pellet that was retained and resuspended in ultra-high purity water. CeO₂NPs without coating were also prepared as followed. 4 nm CeO₂NPs were synthesized by the chemical precipitation of Ce(NO₃)₃ · 6H₂O (Sigma-Aldrich, St. Louis, MO, USA) in a basic aqueous solution ⁴⁶. In this method, 10 mM of Ce(NO₃)₃ · 6H₂O is dissolved in 100 mL of milliQ H₂O at room temperature. Next, 1 mL of tetramethylammonium hydroxide (TMAOH, 1.0 ± 0.02 M in H₂O) is added, and the mixture is left under stirring for 24 hours. Afterwards, purification of NPs is carried out by centrifugation and resuspension in a solution of 1 mM TMAOH, which act as a stabilizer. CeO₂NPs were kept at 4 °C until administration.

Characterization of CeO₂NPs suspension

Transmission Microscopy (TEM) was used to analyze size and morphology of the particles. X-ray diffraction of nanoparticles were analyzed to determine their crystallinity; surface chemistry (Ce⁵⁺/Ce⁴⁺) ratios on the surface of CeO₂NPs was analyzed using X-Ray photoelectron spectroscopy as previously described by Deshpande et al ⁴⁷. The amount of the PVP on the coated CeO₂NPs was quantified by thermogravimetric analysis (TGA), using a PerkinElmer TGA 8000™ Thermogravimetric analyzer, performed under nitrogen flow (60 mL/min) from 40 to 1146 °C, with a 5 °C/min heating rate. Moreover, the optical properties were analyzed using Ultraviolet–Visible Spectrophotometer (PerkinElmer, Lambda 750 S, 60 mm Int. Sphere). The same instrument was used for analysing the optical properties of CeO₂NPs in the biological media. Hydrodynamic diameter and ζ-potential of the CeO₂NPs suspensions in the different assay conditions were measured by Dynamic light scattering (DLS) and electrophoretic light scattering, respectively, using a Zetasizer Nano ZS particle size analyser from Malvern Instruments Ltd. (Malvern, United Kingdom) essentially as described elsewhere ⁴⁸. Colloidal stability was measured both in distilled water and algal culture medium (six times diluted Tris-Acetate-Phosphate, TAP/6⁴⁸).

Biological end-points

The unicellular green alga C. reinhardtii Dangeard (strain CCAP 11/32A mt +) was obtained from the Culture Collection of Algae and Protozoa of Dunstaffnage Marine Laboratory (Scotland, UK). Growth inhibition experiments using C. reinhardtii were performed in TAP/6 medium as described in the standard OECD TG 201⁴⁸. Exposure experiments to CeO₂NPs suspensions were carried out in 12 mL of TAP/6 culture medium in 25 mL flasks. Growth inhibition experiments were performed for 72 h under the same experimental conditions using a set of serial dilutions at least in triplicate. The effect of CeO₂NPs on the growth of microalgae was assessed by measuring the optical density at 750 nm after 72 h of exposure. In vivo fluorescence of chlorophyll a (Ch a) was also measured as a key parameter of photosynthesis status after 24 h of exposure. 100 µL of each sample was transferred to an opaque black 96-well microtiter plate and fluorescence of chlorophyll a was recorded with 485/645 excitation/emission wavelengths on a Synergy HT multimode microplate reader (BioTek, Seattle, WA).

Flow cytometric (FCM) analyses

FCM analyses of C. reinhardtii cells were performed on a Cytomix FL500 MPL flow cytometer equipped with an argon-ion excitation wavelength (488 nm), detector of forward (FS) and side (SS) light scatter and four fluorescence detectors (Beckman Coulter Inc.,

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Fullerton, CA, USA), as described previously. The intracellular reactive oxygen species (ROS), superoxide anion and hydrogen peroxide produced by C. reinhardtii were measured by means of the cell permeable fluorescent dye dihydrodorhamadine 123 (DHR 123) and hydroethidine (HE), respectively. MitoTracker Orange CM-H2TMRos (Invitrogen Molecular Probes) was used to target mitochondria and evaluate possible alterations of mitochondrial ROS homeostasis. The level of cellular lipid peroxidation was evaluated with C12,C16-BODIPY®. Changes in cytoplasmic membrane potential were evaluated with the probe DiBAC4(3). FCM was also used to detect cells with cell membrane damage by using the fluorescent probe Propidium iodide (PI) and cells with altered metabolic activity by using Fluorescein Diacetate (FDA). FDA is a non-polar, hydrophobic, non-fluorescent esterified compound which readily permeates the cell membrane and is hydrolysed by non-specific esterases, leaving the fluorescent by-product fluorescein. Therefore, fluorescein is accumulated by active cells and it has been previously shown as a rapid and effective technique to assess microalgal metabolic activity. Data acquisition was performed using MXP-2.2 software, and the analyses were performed using the Flowing 2.5.1 software. Fluorescence was analysed in Log mode. Cells were incubated with the appropriate fluorochrome for each parameter at room temperature and in the dark, after 24 h of CeO2NPs exposure, prior to FCM analyses. All fluorochrome stock solutions were prepared in dimethyl sulfoxide (DMSO) and stored at −20 °C, with the exception of the solution of propidium iodide (PI), which was made in ddH2O and stored at 4 °C. The fluorochrome concentrations and incubation times were as reported elsewhere and can be found in the Electronic Supplementary Information (ESI) Table S1. Three independent experiments with triplicate samples were carried out for each parameter.

Internalization studies

In order to determine the amount of CeO2NPs inside cells, the algal cells were incubated with 10 mg/L CeO2NPs for 12 h and 48 h. Then, they were collected by centrifugation (4000 rpm) and the supernatant was recovered to determine the amount of free suspended CeO2NPs. 20 mM EDTA was used to remove the CeO2NPs bound onto the cell wall and the samples were again centrifuged. This process was repeated three times and the supernatant was used to calculate the content of CeO2NPs measured as Ce bound to the algal cells (this method was certainly effective as no nanoparticles attached to the cell envelopes were observed nor detected by Transmission Electron Microscopy coupled with X-Ray Energy Dispersive Spectroscopy (TEM-XEDS) after the washing steps, see ESI Figure S1; however, it is worth noting that the presence of Ce at very low concentrations cannot be discarded as there might be still some Ce that could not be detected by this technique due to its detection limit). The remaining algal pellet was acid-digested for 12 h to calculate the intracellular Ce. The Ce content in all samples was measured by Inductively Coupled Plasma Mass Spectrometry (ICP-MS) on an ICP-MS NexION 300XX from Perkin-Elmer. Moreover, in other set of experiments, algal cells were incubated 30 min with sodium azide (NaN3; 0.25 mM), monodansylcadaverine (MDC; 0.2 mM), 5-(N-ethyl-N-isopropyl)-amiloride (EIPA; 10 μM) and filipin complex during 30 min before NP exposure in order to block the different endocytic routes and evaluate the impact of endocytosis on CeO2NPs internalization and toxic effects through the photosynthetic parameter of Chl a fluorescence. A time period of 12 h was chosen to assess the endocytosis of algae under a healthy condition as described by Wang et al. ICP-MS was also used for the intracellular determination of CeO2NPs after the treatment with MDC. For TEM analysis, algal cell suspensions exposed to CeO2NPs before and after EDTA treatment were collected by centrifugation (4000 rpm during 4 min) and prepared as described elsewhere. Briefly, cells were fixed with glutaraldehyde (3.1%; phosphate buffer, pH 7.2) in agar blocks for 3 h at 4 °C. Post-fixation was performed with osmium tetroxide in phosphate buffer for 2 h at 4 °C. Samples were dehydrated in ethanol and embedded in Durcupan resin and, then, sectioned in a Leica Reichert Ultracut S ultramicrotome, stained with uranyl acetate 2%. Ultrathin sections were visualized on a JEOL (JEM 1010) electron microscope (100 kV) or on a JEOL JEM 2100 (200 kV) coupled with XEDS. All reagents used for TEM preparations were Electron Microscopy grade.

Gene expression

Gene expression studies were carried out by the Genomics Unit of the Madrid Science Park (Madrid, Spain). Briefly, total RNA from control and CeO2NPs samples were extracted from frozen cell pellets using RNeasy Mini kit (Qiagen). Remaining genomic DNA was removed using RQ1 RNase-Free DNase (Promega) for 30 min at 37 °C. The concentration of RNA was spectrophotometrically determined in a Nanodrop (Thermo Scientific). First-strand cDNA was synthesized from 250 ng of total RNA using the High Capacity cDNA Archive Kit (Applied Biosystems, Thermo Fisher), Real-time PCR of selected genes was performed using SYBR Green in an AB fast-7900HT System (Applied Biosystems, Thermo Fisher) under standard running conditions. The selected genes were 16S, a constitutively expressed control gene, and CHC1, a gene in C. reinhardtii thought to be involved in clathrin-mediated

**Table 1: Physicochemical properties of the tested CeO2NPs in algal culture medium.**

<table>
<thead>
<tr>
<th>Sample Name</th>
<th>PVP chain length (Da)</th>
<th>ζ-Pot (mV)</th>
<th>DLS Diameter (nm)</th>
<th>PDI*</th>
</tr>
</thead>
<tbody>
<tr>
<td>CeO2NP</td>
<td>-</td>
<td>-13.0 ± 1.1</td>
<td>10.2 ± 0.5</td>
<td>0.38</td>
</tr>
<tr>
<td>CeO2NP10</td>
<td>10k</td>
<td>-8.1 ± 0.8</td>
<td>6.0 ± 0.1</td>
<td>0.18</td>
</tr>
<tr>
<td>CeO2NP40</td>
<td>40k</td>
<td>-7.3 ± 0.4</td>
<td>8.2 ± 0.6</td>
<td>0.15</td>
</tr>
<tr>
<td>CeO2NP360</td>
<td>360k</td>
<td>-9.2 ± 0.3</td>
<td>12.5 ± 0.1</td>
<td>0.28</td>
</tr>
</tbody>
</table>

*Polydispersity Index

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endocytosis\textsuperscript{39}. RT-qPCR primer used in this study were 185f (GCTAGTTAAGCGGAGTCTAT), 185r (AGCCAAAGCTCAATCCGAACA), CH1f (CCGCCGGATCGTCCCTCT) and CH1r (TGCCTCGCTCTGTTTTGTTGTC), which were designed using the DNASTAR's Lasergene software. The $2^{-\Delta\Delta C_{T}}$ scheme was used to normalize and calibrate transcript values relative to the 18S gene using RQ Manager Software (Applied Biosystems)\textsuperscript{60}.

**Statistical Analysis**

Means and standard deviation values were calculated for each treatment from three independent replicate experiments. Statistical analyses were performed by using R software 3.0.2 (The R Foundation for Statistical Computing\textsuperscript{61}) and Rcmdr 2.0–4 package. A one-way ANOVA coupled with Tukey's HSD (honestly significant difference) post-hoc test was performed for comparison of means. Statistically significant differences were considered to exist when $p < 0.05$.

**Results**

**Physicochemical characterization of CeO$_2$NPs**

Three CeO$_2$NPs were synthesized with three different PVP molecular weight coatings (10 kDa, 40 kDa and 360 kDa). Accordingly, the CeO$_2$NPs were named CeO$_2$NP10, CeO$_2$NP40 or CeO$_2$NP360, depending on the PVP chain length. CeO$_2$NPs without coating were referred to as CeO$_2$NP. A complete characterization of all CeO$_2$NPs used regarding size, morphology, crystallinity, optical properties, coating measurements and surface chemistry can be found in ESI Figure S2. CeO$_2$NP, CeO$_2$NP10, CeO$_2$NP40 and CeO$_2$NP360 had spherical shape with diameters approximately of 5, 5, 7 and 12 nm, respectively. Regarding crystallinity, it was not possible to add this information for capped CeO$_2$NP as the PVP capping agent interfered with the XRD measurements. However, it has been previously shown that CeO$_2$ nanorods and nanocubes have particular structure with [100] and [110] + (100) facets, respectively\textsuperscript{62,63}, while spheres of CeO$_2$NPs show similar crystallinity properties, exposing the characteristic diffraction peak at $2\theta = 28.51^\circ$ which correlate to (111) crystal plane\textsuperscript{64}. ESI Figure S2 also illustrates UV-visible absorbance spectra of all CeO$_2$NPs. NP surface chemistry (Ce$^{3+}$/Ce$^{4+}$ ratio) has been also included, since this property, among other important nanoparticle characteristics, has been recently revealed as a key factor of uncoated CeO$_2$NPs toxicity\textsuperscript{38}. CeO$_2$NP40 had the highest % surface Ce$^{3+}$ (100 %) followed by CeO$_2$NP360 (81.3 %), while CeO$_2$NP and coated CeO$_2$NP10 showed similar % surface Ce$^{3+}$ with 32 and 39 %, respectively. Table 1 shows the physicochemical characteristics of the four CeO$_2$NPs at 10 mg/L suspended in the algal culture medium (pH 7). The particles had a negative surface charge ($\zeta$-potential) in TAP/6 with similar values from -7 to -13 mV. There was a slight increase in the effective diameter based on the PVP-chain length: 6.0 nm, 8.2 nm and 12.5 nm for CeO$_2$NP with 10 kDa-PVP, 40 kDa-PVP and 360 kDa-PVP, respectively; whereas the uncoted CeO$_2$NPs had a diameter size of 10.2 nm.

**Toxicity of the CeO$_2$NPs towards C. reinhardtii: effect on growth and photosynthesis**

The effect of the coated and uncoated CeO$_2$NPs towards C. reinhardtii is shown in Figure 1. Figure 1A shows the biological effect of 72 h exposure to CeO$_2$NPs on the growth of C. reinhardtii in the 0.1-50 mg/L range. CeO$_2$NP was more toxic at low concentrations (Minimum Inhibitory Concentration (MIC): 5 mg/L) than PVP-coated CeO$_2$NPs, irrespective of PVP molecular weight. However, CeO$_2$NPs10 and CeO$_2$NPs40 resulted in higher growth inhibition at 50 mg/L. As photosynthesis is a key process in this organism, we checked whether the photosynthetic machinery of C. reinhardtii was affected by CeO$_2$NPs by measuring the in vivo chlorophyll $a$ (Ch $a$) fluorescence emission. There was a statistically

Figure 1. Effect of 72 h exposure to the four CeO$_2$NPs on the growth (A) and in vivo Ch a fluorescence of C. reinhardtii. Statistically significant differences ($p < 0.05$) are marked by asterisks. Ch a: chlorophyll a. Control values of Ch a fluorescence were in the range of 944.6 to 1094.1 arbitrary units (a. u.).
significant increase in the Ch a fluorescence of all samples with respect to control levels clearly dependent on NP concentration. In this regard, the increase observed by all CeO₂NPs has been related with an interruption of the electron transport at the acceptor side of the Photosystem II[55, 66]. Interestingly, while PVP-CeO₂NPs induced photosynthetic alterations at concentrations as low as 1 mg/L, CeO₂NP did not cause any statistically significant effect until 10 mg/L. The toxicity of PVP without NPs and TMAOH (the agent used for the stabilization of uncoated NPs) was also investigated to account for any toxic effect. Concentrations higher than 100 mg/L and 2.5 mM, respectively, were needed to induce a noticeable growth inhibition effect, supporting the previous results regarding the low toxic profile of these compounds[57, 66] (ESI Figure S3 and S4). The results of growth inhibition together with the changes induced in the photosynthetic system suggested that coated and uncoated CeO₂NPs may display different biological toxic mechanisms to C. reinhardtii.

The effects of CeO₂NPs on relevant cellular biomarkers

Therefore, in order to clarify the mechanisms underlying the observed toxicity, several cytotoxicity biomarkers (cell membrane integrity, oxidative stress, metabolic activity and cytoplasmic membrane potential) were analysed by FCM after 24 h of exposure. Two different concentration of CeO₂NPs were chosen: the highest predicted environmental concentration (as calculated by O’brien et al (2011), 0.1 mg/L,[69]) and also an effective NP concentration (10 mg/L), i.e. the concentration at which all CeO₂NPs significantly increased Ch a fluorescence.

The highest predicted environmental concentration of 0.1 mg/L did not produce any alteration in the analysed physiological parameters (see ESI Figure S5 and Figure S6). However, several alterations were observed with a concentration of 10 mg/L. To check whether the cytoplasmic membrane was damaged by CeO₂NPs, the fluorescent dye PI was used. The flow cytograms showed the presence of two distinct cell subpopulations, R1 and R2, in control cultures (Figure 2A). R1 comprised around 89 % of total cells and corresponded to intact cells (intact membranes). R2 subpopulation comprised cells with a basal level of damaged membranes (around 11 % of total cells). FCM results showed that only CeO₂NP significantly (p < 0.05) affected membrane integrity by increasing the R2 subpopulation to 21 %, indicating a clear damage to the cytoplasmic membrane. As uncoated CeO₂NPs were suspended in TMAOH, the effect of TMAOH on cytoplasmic membrane was also tested (ESI Figure S7). Neither TMAOH nor coated-CeO₂NPs displayed alterations in cytoplasmic membrane integrity.

The effect of CeO₂NPs on cytoplasmic membrane potential of C. reinhardtii was also studied by FCM using the fluorescent dye DiBAC₄(3) (Figure 2B). Flow cytograms of control cells showed two clear subpopulations (R1 and R2). R1 was the largest one comprising around 96 % of total cells while the R2 subpopulation (showing a slight membrane depolarization) accounted for 4.2 % of total control cells. CeO₂NP, CeO₂NPs10 and CeO₂NPs40 considerably increased membrane depolarization by 13.1 %, 27.4 % and 36.9 % of total cells (R2 subpopulation; Figure 2B), respectively. CeO₂NPs360 did not have any significant effect on this parameter. Furthermore, as shown in Figure 2C, the percentage of metabolically non-active cells (R2), determined by flow cytometry using FDA, increased significantly (p < 0.05) in cultures exposed to all treatments, in comparison with control levels. However, CeO₂NPs10 and CeO₂NPs40 had the highest percentages with 34 % and 35 % of total cells with a lower metabolic activity.

The production of intracellular ROS in exposed cells has been identified as an important toxicity mechanism for CeO₂NPs[55, 70]. The fluorescent indicators HE and DHR123 were used to determine intracellular superoxide anion (O₂⁻) and H₂O₂, respectively (Figure 3A and 3B). The CeO₂NPs coated with PVP (CeO₂NPs10, CeO₂NPs40 and CeO₂NPs360) caused a significant increase in the intracellular level of both oxidant species, while CeO₂NP did not produce any alteration. CeO₂NPs40 led to the highest level of intracellular hydrogen peroxide formation (Figure 3B). The alterations of
mitochondrial ROS homeostasis and cellular lipid peroxidation were revealed by MitoTracker-selective probe and C6-C7-BODIPY– fluorescent dye, respectively. As shown in Figure 3C, ROS formation in mitochondria increased in all treated cells with the highest level of damage being produced by CeO2NPs40, followed by CeO2NPs10, CeO2NPs360 and CeO2NP. Regarding cellular lipid peroxidation, statistically significant differences were only detected for CeO2NPs40 (Figure 3D).

Taking these results together, we observed a different toxicological pattern between coated and non-coated CeO2NPs. Uncoated NP markedly damaged the algal cytoplasmic membrane, leading to limited intracellular effects. Contrarily, PVP coated CeO2NPs caused severe intracellular damages mediated by an increase in the number of metabolically non-active cells and a strong disbalance in oxidative stress. The fact that they induced those effects without affecting directly the cytoplasmic membrane integrity may indicate that PVP-CeO2NPs internalize, possibly by using the internalization routes described for other eukaryotic organisms.71

Internalization of CeO2NPs

TEM was used in order to prove the effectiveness of the EDTA washing steps, but also, to find direct evidence of CeO2NPs internalization (see ESI Figure S1), however, we were not able to observe the precise moment when endocytosis occurred. To investigate the internalization of CeO2NPs from a different perspective, the distribution of cerium in the culture medium (Figure 4A and D), adsorbed to the cell wall (Figure 4B and E) and measured inside the cells (Figure 4C and F) was analyzed by ICP-MS after 12 h (Figure 4A-C) or 48 h (Figure 4D-F) of exposure. There were no differences in the cerium content in the culture medium after 12 h of exposure (Figure 4A). However, uncoated NPs led to more cerium attached to the cell wall than PVP coated NPs (Figure B), with much less inside cells (Figure C). No differences were found among CeO2NPs10, CeO2NPs40 or CeO2NPs360. Different behavior was observed after 48 h of contact between CeO2NPs and algal cells. CeO2NP appeared mostly in suspension as compared to the other NPs (Figure 4D), but PVP coated NPs preferentially attached to the cell wall. Also, CeO2NP internalized in a higher amount than the other NPs after 48 h. The differences found in the internalized NPs after 12 and 48 h of exposure (Figure 4C and 4F) might be related with the different interaction of CeO2NPs with cytoplasmic membrane. On the one hand, PVP coated CeO2NPs, considering that they interact with the cytoplasmic membrane without damaging it, may trigger some endocytic mechanisms, which usually take place in short periods of time, increasing the amount of internalized NPs at early stages (12 h) in comparison to uncoated NPs. On the other hand, the fact that CeO2NP seriously damaged the cytoplasmic membrane suggests that these NPs could directly pass through membrane holes, preferentially accumulating inside the cells at higher exposure time (48 h). Although FCM analyses were performed at 24 h and the internalization studies at 48 h, the results obtained with those different approaches have given different but complementary information which can be used to link the complexity of the effects of CeO2NPs. Furthermore, as internalization studies with environmentally relevant organisms are not yet fully studied, some processes such as NP exocytosis or, even, particle internalization blocked by saturation cannot be discarded at long periods of time.

Endocytosis inhibitors and potential route of uptake

As internalization was observed for all CeO2NPs, different endocytosis inhibitors were used in order to elucidate the uptake route involved. MDC, filipin complex, EIPA and NaN3 were used to block the clathrin and caveolin dependent endocytosis, macropinocytosis and energy dependent endocytosis, respectively. These compounds did not cause any harmful effect at the concentrations used in this study (see ESI Figure S8). As stated above, photosynthesis was affected by all NPs (section 3.2), so algal Ch fluorescence was used as a key biological parameter in order to assess the effect of inhibitors on NP internalization toxic effects. It was considered that whether an inhibitor efficiently blocked the entry of CeO2NPs through a specific endocytic route, then the toxicity related with photosynthesis should be reduced, so that the level of Ch fluorescence remained as control values. As shown in Figure 5, only the inhibitor associated with clathrin dependent endocytosis, MDC, was able to effectively block the entry of CeO2NP, CeO2NPs10 and CeO2NPs360 as they were not able to induce any alteration on Ch fluorescence. This was further corroborated as the level of CeO2NPs inside cells after the MDC treatment was calculated by ICP-MS and supported the results shown before as MDC reduced the entry of CeO2NP, CeO2NP10,
ceo2NP40 and CeO2NP360 by 17.2%, 58.3%, 35.9% and 22.9%, respectively (see ESI Figure S9), in agreement with previous work showing that inhibitors may not block the entry of NPs completely. The results suggested that the entry of PVP-CeO2NPs in algal cells may be only through endocytosis, whereas CeO2NP could enter the cells both by direct damage to the cytoplasmic membrane and by endocytosis processes as has been demonstrated with these internalization experiments. The results found here also suggest that CeO2NPs uptake in C. reinhardtii may be mediated by clathrin-dependant endocytosis.

**Effect of CeO2NPs on transcription of the CHCI gene**

As clathrin-dependant endocytosis was revealed as the main endocytic route of CeO2NPs internalization, RT-qPCR was used to determine changes in the expression of CHCI; a gene in C. reinhardtii thought to be involved in clathrin-mediated endocytosis. It is worth noting that the present work is the first aiming at attempting a genetic approach on endocytosis in this organism as no experimental evidence on the role of this gene is available up to date. Though little is known about endocytosis in C. reinhardtii, clathrin-coated vesicles have been purified from both wild type and a wall-less mutant cells, supporting genetic evidence of clathrin-mediated endocytosis in this organism. Figure 6 shows the effect of CeO2NPs on expression of this gene. At a short exposure time of 1 h, CHCI expression was upregulated by 31.7%, 32.7%, 33.2% and 69.1% for CeO2NP, CeO2NP40, CeO2NP10, respectively. However, there were no statistical differences at longer exposure time (4 h; ESI Figure S10), indicating that NP internalization process starts on first contact between algal cells and NPs, particularly for PVP-coated CeO2NPs with increased internalization after only 12 h of exposure (Figure 4C).

**Discussion**

Due to their unique properties, CeO2NPs have a number of interesting current and potential future applications that make them one of the most promising nanomaterials. However, their widespread use may increase their presence in the environment.

**Fig. 4.** Analysis of the Ce content in three different compartments after 12 h (A-C) and 48 h (D-F) of exposure to CeO2NP, CeO2NP10, CeO2NP40 and CeO2NP360. (A and D) The Ce content as CeO2NPs adsorbed to the algal envelopes. (C and F) The Ce content inside cell as internalized CeO2NPs.

**Fig. 5.** Analysis of the effect of CeO2NPs (CeO2NP, CeO2NP10, CeO2NP40 and CeO2NP360) and inhibitors (MDC, EIPA, NaN3, and filipin complex) on Ch a fluorescence of C. reinhardtii. CT: control. Statistically significant differences (p < 0.05) are marked by asterisks.
Based on the results presented here, the actual highest predicted environmental concentration did not cause any adverse effect on C. reinhardtii nor in terms of microalgal growth inhibition or in the analyzed physiological parameters. Therefore, the environmental risk of CeO₂NPs may be low according to the available data, although it should be emphasized that chronic exposure even at low concentration may cause adverse effects and merits further studies. However, in order to define the actual hazard of CeO₂NPs, it will be necessary to determine the real concentration of these nanoparticles in the environment or to create probabilistic models which improve the current knowledge and to test those concentrations in a battery of environmentally relevant organisms. From a mechanistic point of view, we have found evidences that CeO₂NPs may be internalized by algae cells and provoke a strong toxic response in the green alga C. reinhardtii, mediated by an increase of intracellular ROS or by direct damage to the cytoplasmic membrane. Several studies have previously reported that CeO₂NPs are toxic to algae and other aquatic organisms. Mechanistic studies, however, are scarce. Here, a combined approach of cellular, physiological and genetic analyses has provided novel insights into toxicological and internalization mechanisms of coated and uncoated CeO₂NPs in a model aquatic organism. As far as we know, this is also the first time that internalization and toxic mechanisms could be related and associated to different types of CeO₂NPs. However, it is worth noting that some non-internalized NPs may also have an impact on the overall toxicity. They may affect the cell envelopes and trigger an intracellular toxic response as previously shown for uncoated CeO₂NPs by means of the interference with the nutrient transport function of the membrane or through ROS generation and oxidative stress induction.

PVP-CeO₂NPs exerted toxicity by means of a different toxicological mechanism than uncoated CeO₂NP. According to the results obtained in this work, cellular membrane integrity was only compromised by the uncoated NP, CeO₂NP, (Figure 2A), indicating that the initial effect of CeO₂NP takes place by interaction with the algal cytoplasmic membrane. Conversely, PVP-coated NPs did not affect the cytoplasmic membrane but induced a strong increase in intracellular ROS (both O₂⁻ and H₂O₂; Figure 3A-B), which led to an increase in mitochondrial ROS formation (Figure 3C) and in the level of intracellular lipid peroxidation for the specific case of CeO₂NPs40. There was no significant increase in the intracellular ROS of algal cells exposed to uncoated CeO₂NP, demonstrating that the direct physical damage to the cellular membrane was the main mechanism explaining the observed toxicity for this NP. Nowadays, CeO₂NPs are surrounded by controversy regarding their biological effects; many authors revealed their reactivity and antioxidant properties, which make them useful for biomedical applications. However, other studies showed that these NPs could also behave as toxic compounds to a variety of cell lines and organisms. According to the results reported here, the biological effects of CeO₂NPs should be revisited taking into account the effect of polymeric coatings. Recently, the percentage of surface Ce⁺³ of CeO₂NPs has been revealed as a key factor which drives the toxicity of uncoated CeO₂NPs. However, this property may be of lower importance if CeO₂NPs are covered with capping agents as the uncoated CeO₂NPs and coated CeO₂NP10 used in this study exhibited similar % of surface Ce⁺³, but they displayed completely different toxicological mechanisms. Moreover, it has been shown that the reactivity of CeO₂NPs may reduce while increasing the thickness of different types of polymeric coatings, therefore the type and thickness of polymeric coating is an important factor in particle reactivity. The results showed here support this hypothesis as CeO₂NPs 360 with a coating of 360 kDa induced a lower extent of biological effects in comparison with the other coated particles with 10 kDa and 40 kDa PVP.

Our results showed that the PVP coating of CeO₂NPs influence the internalization kinetics. After 12 h, internalization was found mostly for PVP-coated NPs while after 48 h the highest level of Ce was found for CeO₂NP. This suggests that as membrane damage increases with time, CeO₂NP may pass through more easily and accumulate inside cells (Figure 4F), explaining their presence in higher amount at later exposure times. PVP-coated CeO₂NPs also interacted with the cellular membrane, but without damaging it and being adsorbed at higher amount to the cell envelopes after 48 h of exposure (Figure 4E). The reasonable explanation of these findings is that PVP coating protected cells from the direct contact with the NP core, but at the same time promoted NP internalization during the early stages of exposure (Figure 4C and Figure 6). The coating itself may enhance the interaction with cells by attaching to certain specific groups at the cytoplasmic membrane. In the same way as bare NPs are surrounded by different biomolecules, forming the so-called protein corona or eco-corona; PVP-coated NPs by
enhancing the interaction with cellular envelopes and triggering endocytic processes\textsuperscript{81-86} could behave similarly.

NP internalization has been recently observed in \textit{C. reinhardtii} for QdTe/CdS Quantum Dots\textsuperscript{87}, PAMAM-dendrimers\textsuperscript{88} and for Ag\textsuperscript{41}, CeO\textsubscript{2}\textsuperscript{40} and CuO\textsuperscript{42} NPs. However, works studying the mechanism of entry in cells are scarce. Many works have studied the machinery related to endocytosis in higher plants\textsuperscript{89, 90}. However, little is known about this process in algae. It has been shown that there exist several routes of endocytosis in eukaryotic organisms\textsuperscript{71} that explain the internalization of nanomaterials in several environmentally relevant organisms\textsuperscript{58, 91-93}. Wang et al.\textsuperscript{92} showed that CdTe Quantum Dots could enter the freshwater alga \textit{Ochromonas danica} directly through macropinocytosis. Moreover, Hoefliger et al. reported that clathrin-dependant pathway played an important role in the endocytosis process in a multicellular green alga (\textit{Chara australis})\textsuperscript{94}. The genomic mapping of \textit{C. reinhardtii} together with some experimental studies have revealed that this microalgae possesses a clathrin-mediated endocytosis pathway\textsuperscript{58, 71, 96} and also that ceria NPs can be internalized\textsuperscript{90}. In the present study, we have shown that clathrin-dependent endocytosis is the main route of CeO\textsubscript{2}NPs entry into the cells of \textit{C. reinhardtii} as MCD (molecule that stabilizes clathrin-coated pits) blocked the entry of all CeO\textsubscript{2}NPs, avoiding their toxic effects related with the photosynthetic apparatus. However, it is worth noting that, in general terms, the internalization of NPs may only occur under certain circumstances. First, particle size is an issue. It has been shown that the cell wall constitutes the first barrier with a pore diameter in the 5-20 nm range\textsuperscript{97}, so internalization of larger or agglomerated particles seems rather improbable, unless they enter (i) via the apical zone where the presence of flagella causes an absence of cell wall\textsuperscript{98} or (ii) during the reproduction period when the newly synthesized cell walls are more facile to penetrate\textsuperscript{97}. Second, as PVP-CeO\textsubscript{2}NPs were able to solely internalize through endocytosis mechanisms, the use of capping agents might also play an important role, enhancing and triggering the endocytic system after interacting with the cytoplasmic membrane.

Several authors have previously shown that the dissolution of CeO\textsubscript{2}NPs may be enhanced at the bio-nano interface and promote the entry of Ce\textsuperscript{3+} ions more easily\textsuperscript{97, 99}. Ma et al\textsuperscript{99} showed that the physicochemical interaction between the NPs and root exudates at the nano-bio interface is the required condition for the transformation of CeO\textsubscript{2}NPs in plant systems. The plant exudates (including organic acids) are known to alter the surrounding environment, impact the rhizobial community, support beneficial symbioses, alter the chemical and physical properties of the soil, among others\textsuperscript{100}. Different studies have shown that exudates could also interact with nanomaterials, promoting NP dissolution\textsuperscript{100-103}. This seems unlikely in the case of \textit{C. reinhardtii} as the release of exudates and organic acids are quite more limited in algal cells in comparison with the plethora of compounds released by plants\textsuperscript{104, 105}. Moreover, the specific physicochemical characteristics of NPs are of crucial importance for determining the reactivity of CeO\textsubscript{2}NPs. Xie et al\textsuperscript{98} used rod-CeO\textsubscript{2}NPs which are NPs with higher concentration of surface defects, and, thus, higher reactivity rates in comparison with other morphologies\textsuperscript{106, 107}. Here, the used CeO\textsubscript{2}NPs were spheres coated with PVP, so the surface reactivity may be limited due to the shielding effect of the coating.

Despite the limited available information about endocytosis in plant cells in comparison with the existing grounded-knowledge for animal cells, different works have highlighted the importance of this process as a crucial mechanism by which plants cells internalize extracellular and plasma membrane material\textsuperscript{108, 109}. Several authors have also underlined its role in the well-functioning of the cells, specifically how clathrin dependent endocytosis is involved in key physiological processes\textsuperscript{110, 111}. Here, using \textit{C. reinhardtii} as model of study, we have found that CeO\textsubscript{2}NPs generated an overexpression of CHC1, gene involved in the clathrin-mediated endocytosis. If this phenomenon implies a biological significance is unknown since cerium is not an essential nutrient for microalgae. Besides, whether nanoparticle internalization is performed to alleviate the impact of CeO\textsubscript{2}NPs in the cellular envelopes or because they obtain an unknown benefit or whether they are mistakenly introduced has to be deeply studied in the future.

As far as we know, based on inhibitors and gene expression analysis, this is the first time that it is shown that clathrin-dependant pathway is the main route of entry of nanoparticles into \textit{C. reinhardtii} cells. Further research is still needed for determining the effect of other coatings in CeO\textsubscript{2}NPs uptake and also, the optimal PVP-chain length that could favour the internalization of NPs. More research is still needed to fully understand the NP internalization (in terms of genes involved and timing of the process) by endocytosis in the green alga.

Conclusions

In this work, we have reported a thorough study on the toxicological effects of uncoated and PVP-coated CeO\textsubscript{2}NPs to \textit{C. reinhardtii}. Monodispersed NPs of cerium oxide and PVP-coated cerium oxide were synthesized and physicochemically characterized in the exposure media. Overall, we conclude that the risks to \textit{C. reinhardtii} posed by the uncoated and coated CeO\textsubscript{2}NPs used here at the predicted environmental concentrations are low, although it should be emphasized that chronic exposure even at low concentration may cause adverse effects and merits further studies. From a mechanistic point of view using higher concentrations, the toxicity of the bare CeO\textsubscript{2}NP took place by damage to cytoplasmic membrane, which eventually caused a decrease in metabolic activity. PVP-coated NPs exerted their toxicity by intracellular ROS formation without direct damage to the cell membrane. Different degrees of cell damage were found depending on PVP chain length, being CeO\textsubscript{2}NPs\textsuperscript{40} the most toxic NP, followed by CeO\textsubscript{2}NPs\textsuperscript{10}. Moreover, internalization evidences were found for all NPs, but, after 48 h of exposure, CeO\textsubscript{2}NP was internalized in higher proportion than PVP-coated CeO\textsubscript{2}NPs, which predominantly appeared attached to the algal envelopes. The studies performed using endocytosis inhibitors indicated that clathrin-dependent endocytosis was the main pathway for NP entry. As internalization
is a complex process with little available information for environmental organisms such as algae, different qualitatively and quantitatively techniques as well as diverse experimental time scales have been performed to better understand the interaction between CeO₂NPs and C. reinhardtii. The results shown here highlight the importance of assessing the toxicity of CeO₂NPs as they are intended to be used and found in the environment due to the toxicological mechanism could be significantly different, depending on NP coating. As discussed above, the surface coating totally changes the interaction of nanomaterials with the environment, so there is still a long way to get a complete picture of the effects of coated nanomaterials in living organisms. Nonetheless, the information presented here will be useful for shedding light regarding the contradictory effects of CeO₂NPs that have been reported in the scientific literature and for the synthesis of safer-by-design nanomaterials.

Conflicts of interest
There are no conflicts to declare.

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