Antioxidant Activity and Toxicity Study of Cerium Oxide Nanoparticles Stabilized with Innovative Functional Copolymers

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Oxidative stress, which is one of the main harmful mechanisms of pathologies including ischemic stroke, contributes to both neurons and endothelial cell damages, leading to vascular lesions. Although many antioxidants are tested in preclinical studies, no treatment is currently available for stroke patients. Since cerium oxide nanoparticles (CNPs) exhibit remarkable antioxidant capacities, the objective is to develop an innovative coating to enhance CNPs biocompatibility without disrupting their antioxidant capacities or enhance their toxicity. This study reports the synthesis and characterization of functional polymers and their impact on the enzyme-like catalytic activity of CNPs. To study the toxicity and the antioxidant properties of CNPs for stroke and particularly endothelial damages, in vitro studies are conducted on a cerebral endothelial cell line (bEnd.3). Despite their internalization in bEnd.3 cells, coated CNPs are devoid of cytotoxicity. Microscopy studies report an intracellular localization of CNPs, more precisely in endosomes. All CNPs reduce glutamate-induced intracellular production of reactive oxygen species (ROS) in endothelial cells but one CNP significantly reduces both the production of mitochondrial superoxide anion and DNA oxidation. In vivo studies report a lack of toxicity in mice. This study therefore describes and identifies biocompatible CNPs with interesting antioxidant properties for ischemic stroke and related pathologies.

1. Introduction

Oxidative stress results from an imbalance between the production of reactive oxygen/nitrogen species (ROS/RNS) and endogenous antioxidant systems. Oxidative stress is a major contributor to the pathogenesis of ischemic stroke (due to the occlusion of a brain vessel).[1–3] Besides neurons, post-ischemic oxidative stress also contributes to endothelial cell damages, leading to vascular lesions and subsequent cerebral hemorrhages, which are associated with a poor prognosis for stroke patients.[4] Although many antioxidants have been tested in preclinical studies and, for a few of them, in clinical trials, there is no antioxidant treatment currently available for stroke.[5–8] Cerium oxide nanoparticles (CNPs), also called nanoceria, hold wide-ranging antioxidant capacities. They indeed scavenge both ROS and RNS such as hydroxyl radical (HO•)[9] and nitric oxide (•NO)[10] while mimicking the antioxidant enzymes superoxide dismutase (SOD) and catalase (CAT).[11–15] Interestingly, the antioxidant activity of CNPs has been associated with a protective effect not only on neurons,[16,17] but also on endothelial cells.[18]
Beneficial effects of CNPs have been reported in preclinical models of various pathologies such as cancer,[19–22] cardiovascular injuries[23,24] or diseases of the central nervous system[16,25] such as Alzheimer’s disease.[26] With regards to brain ischemia, neuroprotection by CNPs has been first shown ex vivo on hippocampal slices submitted to hypoxia.[27] In a rat model of focal cerebral ischemia, CNPs were shown to reduce ROS production and the lesion’s volume.[28] More recently, CNPs either loaded with an antioxidant, edaravone, or combined with a targeting ligand efficiently decreased the outcomes of ischemic strokes in rats.[18,29,30]

Despite these encouraging results, CNPs suffer from certain limitations. Their clinical translation requires a significant improvement of their surface properties, particularly through the development of an appropriate functionalization. Control of interfacial properties is a central issue in nanoparticle’s development to increase their biocompatibility, stability, and stealthiness in biological media. In particular, uncoated metal oxide nanostructures are unstable in such media,[31] thus leading to a poor pharmacodynamic profile. In addition, nanoceria’s coating strongly influences cellular uptake and cytotoxicity.[32,33] The objective of our study is to develop coated CNPs displaying increased stability while retaining their antioxidant properties and decreasing their cytotoxicity.

One of the most promising surface functionalization methods makes use of polymers and co-assembly techniques.[34,35] This method takes advantage of the library of polymer architectures and binding agents synthesized polymer and coordination chemistry. Many types of polymers, natural or synthetic have indeed been used on CNPs: citrate/poly(acrylic) acid,[32,33] dextran/poly(acrylic) acid,[36] oleic acid–PAAOA–PMAOA,[37] PSPM–PME–TAC,[38] PVA–PBCA–PGLA,[39,40] DNC–PNC–ANC,[41] and heparin.[42] Recently, our group proposed a coating strategy based on functional statistical graft copolymers where phosphonic acids (a strongly binding agent to metals)[43,44] and poly(ethylene glycol) (PEG) chains are covalently grafted to a poly(methyl methacrylate) backbone. Copolymers with multiple phosphonic acids provide resilient coatings and long-term stability (>months). In addition, they are well suited to a broad range of metal oxide particles, including cerium, aluminum, iron, and titanium oxides.[45] Protein adsorption studies have highlighted that PEG densities around 0.5 nm$^{-2}$ and layer thickness about 10 nm (corresponding to PEG molecular weight of 2000–5000 g mol$^{-1}$) provide excellent serum protein resistance.[46] In vivo magnetic resonance imaging established the potential of phosphonic acid–PEG polymer coatings to significantly prolong the pharmacokinetics of intravenously injected iron oxide nanoparticles in mice.[47] Last but not least, this approach paves the way to use amine terminated PEG terpolymers that can be later covalently functionalized with targeting or imaging moieties.

In this article, we describe the CNPs structural and antioxidant properties, and the functional polymers used as coating. These polymers are statistical copolymers bearing on one hand phosphonic acids for binding to the cerium surface and on the other hand either PEGs or amine modified PEGs to reduce the mononuclear phagocyte system uptake and allow further functionalization.[45,46,48] The impact of the coating on the antioxidant properties of nanoceria was also examined ex vivo. To study the interest of these antioxidant properties in the context of stroke and specifically against vascular lesions, in vitro studies were conducted on cerebral endothelial cells (bEnd.3 cells). We assessed nanoceria toxicity, their cellular uptake and intracellular location in endothelial cells and their effect on ROS production. Finally, we investigated the in vivo toxicity and biodistribution of nanoceria in mice.

2. Results

2.1. Nanoceria Coating and Characterization

2.1.1. Bare Nanoceria Particles

CNPs were synthesized by thermo-hydrolysis of cerium nitrate salt under hydrothermal conditions at acidic pH.[49,50] The nanoceria structure was resolved using a combination of techniques including transmission electron microscopy (TEM), dynamic light scattering (DLS), X-ray photoelectron spectroscopy (XPS), wide-angle X-ray scattering (WAXS) and UV–vis spectrometry. Figure 1a shows a TEM image of CNPs in the form of 2.9 nm crystallite agglomerates. The size distribution obtained leads to a median diameter of 7.8 nm and a dispersity of 0.17. Using static and DLS, the hydrodynamic diameter $D_{h}$ and the molecular weight $M_{w}$ of the particles were found at 9.8 nm and $3 \times 10^{7}$ g mol$^{-1}$.[45,51] Figure 1b displays XPS results obtained from bare CNP powder samples. The Ce3d XPS spectrum is decomposed into five peaks,[52,53] three being associated with the Ce$^{4+}$ ions and two with the Ce$^{3+}$ ions. The XPS data analysis on the peak assignment, on the determination of the binding energies and on the Ce$^{3+}$ fraction is provided in the Supporting Information, S1 and leads to a Ce$^{3+}$-fraction of 14%. Note that with this Ce$^{3+}$ fraction, only one pair of Ce3d peaks (here the U’-V’ indicated in red) is visible in the XPS intensity. The WAXS diffractogram in Figure 1c reveals that the nanoceria have a fluorite-like face-centered cubic structure. From the Rietveld analysis, the lattice constant was derived and found at 0.54151 nm. From the width of the Bragg reflections, the crystallite size was estimated at 2.9 nm. UV–vis spectrometry performed on dispersions at different concentrations between 0.02 and 2 g L$^{-1}$ allowed to retrieve the absorbivity $\epsilon(\lambda)$ as a function of the wavelength (Figure 1d). With decreasing $\lambda$, the $\epsilon$-data reveal a maximum at 290 nm followed by a strong decrease down to 400 nm, in excellent agreement with earlier reports.[28,37,51]

2.1.2. Polymer Synthesis and Characterization

Three statistical copolymers were synthesized through free radical polymerization. The first one (P1) was obtained through the copolymerization of PEG methacrylate with a methacrylic monomer bearing a phosphonate group (MPh). Resulting copolymer is composed of an equimolar ratio (0.50:0.50) of phosphonic acid groups and methyl terminated PEGylated lateral chains. The PEGs have molecular weight 2000 g mol$^{-1}$, leading to the acronyms MPEG$_{2K}$-MPh where “M” refers to methacrylic nature of the co-monomers and “Ph” to phosphonic acid (Figure 2 and Table 1). The synthesis was performed following a procedure previously described.[48,54] The two other polymers (P2 and P3) are terpolymers containing methyl- and amine-terminated PEG
Figure 1. Nanoceria characterization using a) transmission electron microscopy (TEM), b) X-ray photoelectron spectrometry (XPS), c) wide-angle X-ray scattering (WAXS), and d) CeO$_2$ dispersion absorptivity curve obtained from UV–vis spectrometry. Inset: image of a concentrated CeO$_2$ dispersion. The nanoceria used in this work have the face-centered cubic fluorite-like structure and a Ce$^{3+}$ fraction of 14%. The particles are small clusters (arrows in Figure 1a) composed of 2.9 nm crystallites. The cluster size has median diameter of 7.8 nm and a dispersity of 0.17 (the dispersity is defined as the ratio between the standard deviation and the average).

Figure 2. Molecular structures of phosphonic acid-based copolymers and terpolymers synthesized in this work. a) The poly(poly(ethylene glycol)methacrylate-co-dimethyl(methacryloyloxy)methyl phosphonic acid) is a statistical copolymer where the repeating units have lateral methyl terminated PEG$_{2k}$ chains and lateral phosphonic acids in the proportion (0.50:0.50). It is abbreviated as P1: MPEG$_{2k}$-MPh. b) The statistical terpolymer P2: MPEG$_{2k}$-MPEG$_{a1k}$-MPh has for repeating units lateral methyl terminated PEG$_{2k}$ chains, lateral amine terminated PEG$_{a1k}$ chains, and lateral phosphonic acids in the molar proportions (0.35:0.15:0.50). c) As in (b) with lateral amine terminated PEG$_{2k}$ chains and molar proportions (0.07:0.43:0.50). This polymer is abbreviated as P3: MPEG$_{2k}$-MPEG$_{a2k}$-MPh.
chains in addition to the phosphonic acid groups (Figure 2 and Table 1). The amine terminated PEGs have molecular weight of 1000 or 2000 g mol⁻¹, leading to the acronyms MPEG₂K-MPh and MPEG₃K-MPEGₐ₂K-MPh, where “a” refers to the amine terminal group. The proportions of PEGs, amine modified PEGs, and phosphonic acids are (0.35:0.15:0.50) for P2 and (0.07:0.43:0.50) for P3. With a MPEG₃K as a co-monomer, it is expected that functional groups linked to the primary amine will be partially embedded in the PEG₂K brush and be protected from non-specific binding. The polymers’ molecular structures are illustrated in Figure 2. Details on the P2 and P3 terpolymer synthesis and their ⁱH NMR characterizations can be found in the Experimental Section and in Supporting Information, S2. The weight-averaged molecular weights $M_	ext{P}$ were determined from static light scattering using the Zimm plots. The P1, P2, and P3 copolymers are found with a molecular weight $M_	ext{P}$ of 20 300, 39 500, and 29 200 g mol⁻¹, respectively. Assuming a molar mass dispersity $D = 1.8$, the number-averaged molecular weight $M_	ext{N}$ is determined at 11 300, 21 900, and 16 200 g mol⁻¹. From these values, the average number of phosphonic acids was estimated at 5.1, 7.7, and 6.6, confirming the presence of multiple functional groups. These later results are summarized in Table 1.

### 2.1.3. Nanoceria Coating

The coating of nanoceria was carried out at acidic pH (1.5) to prevent aggregation. Moreover, it was found that below the critical mixing ratio of 1.5, well-dispersed coated particles were obtained whilst above, they associate and form large aggregates that eventually precipitate in solution. In this study, the mixing ratio was defined as the mass ratio between particles and polymers in the mixed dispersion. These results are interpreted in the framework of the non-stoichiometric adsorption model we developed in the context of polymer coating. This model assumes that the polymers adsorb spontaneously onto CNPs thanks to the phosphonic acid groups anchoring at the surface. The association is described as non-stoichiometric because the number of polymers adsorbed per particle depends on the mixing ratio. DLS was used to measure the polymer thickness $h$. For the three polymer coats, we found $h = 9.1$, 10.2, and 11.2 nm (Table 2). These values are consistent with stretched PEG chains forming a polymer brush. From the critical mixing ratio, the PEG density can be estimated, resulting in 0.3, 0.2, and 0.6 nm⁻² for P1, P2, and P3 coating respectively. Electrokinetic measurements using laser Doppler velocimetry and phase analysis light scattering mode show that the bare nanoceria are positively charged with a zeta potential $\zeta = +21$ mV, whereas coated particles are globally neutral (Table 2). Concerning the colloidal stability, earlier studies have shown that nanoceria coated with phosphonic acid PEG copolymers provide resilient coatings and long-term stability, while avoiding protein adsorption (Supporting Information, S3).

### 2.1.4. Antioxidant Properties of Nanoceria

The antioxidant capacities of nanoceria were evaluated ex vitro regarding SOD- and CAT-like activities, to assess the impact of multi-PEG coatings. The SOD-like activity $A_{\text{SOD}}$ is defined as the percentage of dismutated superoxide anions at the end of the assay. Figure 3a shows the $A_{\text{SOD}}$ for bare and coated nanoceria (P1: MPEG₂K-MPh, P2: MPEG₂K-MPEGₐ₂K-MPh, and P3: MPEG₂K-MPEGₐ₂K-MPh) at 1, 10, 100, and 1000 µg mL⁻¹. Our results showed similar dismutation rates between bare and coated CNPs, which increased with concentration. The concentration results are interpreted in terms of a Langmuir-type adsorption isotherm where $A_{\text{SOD}}$ scales with the surface area concentration and with the Ce⁴⁺ fraction. The addition of a polymer coat on the particles did not alter their catalytic activity. The CAT-like activity of nanoceria was investigated at 1, 10, 100, and 1000 µg mL⁻¹. The CAT-like activity $A_{\text{CAT}}$ is defined as the percentage of decomposed H₂O₂ at the end of the reaction. $A_{\text{CAT}}$ increases sharply as a function of the nanoceria concentration and saturates at high dose (1000 µg mL⁻¹). When coated, a 20% reduction of the CAT-like activity of nanoceria is observed compared to bare CNPs, as shown for the data at 10 and 100 µg mL⁻¹. These results demonstrate that phosphonic acid PEG copolymers only slightly affect the CNP biomimetic catalytic activity, while increasing their long-term colloidal stability.

### 2.2. Effect of CNPs on Murine Cerebral Endothelial Cells bEnd.3

#### 2.2.1. Toxicity of Nanoceria

Metabolic activity and mortality of bEnd.3 cells were evaluated after 4 and 24 h incubations with nanoceria (Figure 4). Glutamate, an excitatory neurotransmitter, whose involvement in cerebral

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**Table 1.** Molecular characteristics of the phosphonic acid PEGylated polymers and copolymers synthesized in this work.

| Abbreviation | Polymer | $M_w$ [g mol⁻¹] | $M_N$ [g mol⁻¹] | Anchoring groups per polymer |
|--------------|---------|----------------|----------------|-----------------------------|
| P1           | MPEG₂K-MPh | 20 300 | 11 300 | 5.1 |
| P2           | MPEG₂K-MPEGₐ₂K-MPh | 39 500 | 21 900 | 7.7 |
| P3           | MPEG₂K-MPEGₐ₂K-MPh | 29 200 | 16 200 | 6.6 |

**Table 2.** Polymer coated cerium oxide nanoparticle hydrodynamic diameter $D_h$, polymer thickness $h$, and zeta potential $\zeta$ determined for particles dispersed in the acetate buffer 0.1 M (pH 4.0).

| Nanoparticles | $D_h$ [nm] | $h$ [nm] | $\zeta$ [mV] |
|---------------|------------|----------|--------------|
| CeO₂ bare     | 9.0        | -        | +21          |
| CeO₂@P1       | 27.2       | 9.1      | +1.4         |
| CeO₂@P2       | 29.5       | 10.2     | -1.1         |
| CeO₂@P3       | 31.5       | 11.2     | +5.8         |
Figure 3. Nanoceria superoxide dismutase- and catalase-like activities. The catalytic activity of bare nanoceria and nanoceria coated with P1 (MPEG₂K-MPh), P2 (MPEG₂K-MPEG₈K-MPh), and P3 (MPEG₂K-MPEG₈K-MPh) as a function of their concentrations was assessed against a) superoxide anions and b) hydrogen peroxide. Data are expressed as mean ± SEM.

Figure 4. Effect of nanoceria on metabolic activity and mortality of bEnd.3 cells. Toxicity of nanoceria was assessed measuring a,b) MTT metabolic activity and c,d) trypan blue mortality, 4 (a,c) and 24 h (b,d) after treatments. For the MTT assay (a,b), results are expressed in percentage of absorbance of control cells, and for the trypan blue assay (c,d), results are expressed in percentage of mortality. Glutamate was used at 100 mM. Data are expressed as mean ± SEM, n = 7–15 at 4 h and n = 5–13 at 24 h. *p < 0.01, **p < 0.001 versus control, ##p < 0.01, ###p < 0.001 versus bare nanoceria at 1000 µg mL⁻¹.

Post-ischemic oxidative stress and damage is well established, was used as a positive control for toxic effect: glutamate (100 mM) induced a loss in metabolic activity at both 4 and 24 h (−17% p < 0.01 and −50% p < 0.001, respectively compared to control cells) and an increase in bEnd.3 cell mortality at 24 h ( +14% p < 0.01 vs control) (Figure 4). With regards to CNPs, at lower concentrations (i.e., 10 and 100 µg mL⁻¹), neither the metabolic activity nor the mortality was modified compared with control cells, independently of the nanoparticle tested. At the highest concentration (i.e., 1000 µg mL⁻¹), CeO₂@P1 and CeO₂@P2 reduced the metabolic activity at 4 h (−25% and −26% p < 0.01, respectively), and all CNPs induced a significant loss in metabolic activity at 24 h (p < 0.01 and p < 0.001). However, the metabolic activity of bEnd.3 cells incubated with 1000 µg mL⁻¹ of CeO₂@P1 or CeO₂@P3 during 24 h was significantly higher compared to cells incubated with bare nanoceria at the same concentration, indicating that both P1 and P3 coatings are reducing the toxicity of CNPs. Only bare nanoparticles induced mortality as observed after both 4 and 24 h incubation at the highest concentration (p < 0.001). At this concentration (i.e., 1000 µg mL⁻¹), the mortality observed with CeO₂@P1, CeO₂@P2, and CeO₂@P3 was significantly lower than bare CeO₂ (p < 0.001). The concentration of 1000 µg mL⁻¹ of CeO₂ is high compared to the concentrations used in vitro in the literature, that is, most studies, notably on endothelial cells, used doses below 100 µg mL⁻¹. With regards to the lower concentrations we used, our results are thus consistent with these studies which did not report any toxicity and/or decrease in viability on endothelial cells, primary or derived from cell lines, whether human or animal. At the exception of Turco et al. which reported a slight but significant
Adsorption, internalization, and intracellular location of nanoceria in bEnd.3 cells. a) ICP-OES nanoceria quantification in b.End3 cells. Bare nanoceria and CeO$_2$@P1, CeO$_2$@P2, and CeO$_2$@P3 were incubated at 1, 10, 100, and 1000 µg mL$^{-1}$ during 24 h. b) Flow cytometry was performed using cyanine-labeled CeO$_2$@P2 and CeO$_2$@P3 incubated during 4 or 24 h at 100 µg mL$^{-1}$. Top: percentage of labeled cells, bottom: mean fluorescence intensity per cell. Data are expressed as mean ± SEM, n = 5. *** p < 0.001 versus control, $^*$ p < 0.05, $^{**}$ p < 0.01 versus 4 h. c) Confocal fluorescence microscopy with cyanine-labeled CNPs (100 µg mL$^{-1}$ during 24 h). bEnd.3 cells were fixed and stained for actin (in yellow) and nuclei (in blue), cyanine-labeled CNPs appear in red. d) TEM images of bEnd.3 cells incubated with CeO$_2$@P2 (1000 µg mL$^{-1}$ 24 h). Bottom images, which are closer views of the delimited areas on the first and the second pictures, show CNPs enclosed in an endosome near the nucleus.

2.2.2. Adsorption, Internalization, and Intracellular Location of Nanoceria

Adsorption/internalization of nanoceria in bEnd.3 cells was first examined by quantification of cerium using ICP-OES (inductively coupled plasma-optical emission spectrometry) (Figure 5a). Our results showed that all CNPs associate with bEnd.3 cells independent of the concentration used, indicating that nanoceria are thus interacting with bEnd.3 cells after a 24 h incubation, even at the lowest concentration (1 µg mL$^{-1}$). Whatever the coating, the detected amount of coated CNPs was drastically reduced compared to bare nanoceria (about 100-fold). This result highlights the stealthiness of coated CNPs compared to their bare counterpart. According to the literature and our data, coating strongly influences internalization.[32,33] However, Qiu et al.[38] reported only a slight decrease of cerium internalization with coated particles compared to our data. They only showed a 3-time reduction with a PSPM coating (3-sulfopropylmethacrylate with negative charges) and by 20% with PMETAC coating (2-(methacryloyloxy)ethyl-trimethylammoniumchloride) compared to bare CNPs after a 48 h incubation.[38] These data underline the furtivity provided to the CNPs by our coating technique. Concerning flow cytometry analysis, our results are in line with data from the literature reporting uptake of nanoceria into endothelial cells within the first few hours of incubation.[18]
Finally, when the bEnd.3 cells were treated with glutamate, we did not observe any change in the internalization ratios (data not shown), which suggests that the influence of the coating is not modulated under these oxidative stress conditions. Additional analyses for adsorption/internalization were carried out through flow cytometry and confocal fluorescence microscopy. Considering the results of coated CNPs on cell toxicity, the concentration of 100 µg mL\(^{-1}\) was selected for the following experiments. The primary amine groups were first quantified by spectrofluorometry using fluoresceamine (Supporting Information, S4). Then, amino-PEG coated CNPs, that is, CeO\(_2@P2\) and CeO\(_2@P3\), were labeled with cyanine-5 (Cy5). The amine free particles CeO\(_2@P1\) were used as a control as no Cy5 is grafted on their surface. Flow cytometry analysis showed that cell basal auto-fluorescence was very low and was not modified by CeO\(_2@P1\). Cyanine-fluorescence was detected in more than 95% of the cells incubated with CeO\(_2@P2\)-Cy5 and CeO\(_2@P3\)-Cy5 at 100 µg mL\(^{-1}\) after a 4 h incubation (Figure 5b). With regards to fluorescence intensity, a four to sixfold increase was observed at 24 h compared to 4 h post treatment for CeO\(_2@P2\)-Cy5 and CeO\(_2@P3\)-Cy5, respectively (p < 0.01 and p < 0.05), indicating an increase in CNPs adsorption/internalization over time (Figure 5b).

Concerning the intracellular location, confocal fluorescence microscopy showed internalization of both cyanine-labeled CNPs CeO\(_2@P2\) and CeO\(_2@P3\) in bEnd.3 cells (Figure 5c). Observations at higher magnification indicated a perinuclear location. The localization of CNPs in different cell types has been studied by others using fluorophore-labeled CNPs. All studies showed localization only in the cytoplasm,[18,38,61-63] including in endothelial cells,[18,59] which is consistent with our data. Some of these studies have demonstrated co-localization with endosomes,[38,61,63] lysosomes,[18] mitochondria, or both lysosomes and endoplasmic reticulum.[64]

TEM representative micrographs of cells incubated for 24 h with CeO\(_2@P2\) are presented on Figure 5d. bEnd.3 cells did not show any sign of alteration that might have been induced by particles such as intracellular vacuoles. Moreover, CNPs did not aggregate at the plasma membrane. The lower panels in Figure 5d, which show enlarged views of the micrograph, reveal individual and slightly aggregated particles located only in the compartments attached to the membrane. These compartments are identified as endosomes. Electron microscopy is conventionally used to characterize the shape and size of CNPs; however, only a few used TEM to determine the subcellular location of nanoceria.[24,59,61,62,65] All these studies have shown, as in the present work, a localization of CNPs exclusively in the cytoplasm, and more precisely in vesicles such as endosomes, lysosomes, and phagosomes. Studies have shown that CNPs were internalized by cells through an energy-dependent uptake: endosome-mediated and macropinocytosis pathways.[38,60,64]

### 2.2.3. Antioxidant Effect of Nanoceria on bEnd.3 cells

**Effect of Nanoceria on Total Intracellular ROS Production:** Intracellular ROS production was measured on bEnd.3 cells using the dichlorofluorescein diacetate (H\(_2\)DCFDA) probe. N-acetylcysteine (NAC) was used as an antioxidant benchmark. The effect of nanoceria on ROS production was first assessed on bEnd.3 resting cells (Supporting Information, S5). The antioxidant NAC significantly reduced the basal levels of intracellular ROS by at least 50% at both incubation times (p < 0.01, p < 0.001), allowing us to validate the present ROS detection technique. After a 4 h incubation, only CeO\(_2@P1\) at 100 µg mL\(^{-1}\) and CeO\(_2@P3\) at 1000 µg mL\(^{-1}\) reduced intracellular ROS generation by 22% (p < 0.05) and 21% (p < 0.01) respectively (Supporting Information, S5). After 24 h of incubation, none of the CNPs reduced ROS production. The absence of antioxidant effect in resting cells is not a disadvantage since ROS produced in physiological conditions play a role within the cells, particularly in cell signaling.

The effect of nanoceria on bEnd.3 cells’ ROS production in oxidative conditions was then examined (Figure 6). Cells were treated with glutamate to mimic the massive release of this neurotransmitter during ischemic stroke that leads to oxidative stress. Glutamate significantly increased ROS levels in bEnd.3 cells at both 4 h (+34%; p < 0.001) and 24 h (+44%; p < 0.05). The antioxidant NAC significantly reduced glutamate-induced ROS production by about twofold (p < 0.001) at both incubation times. All nanoceria induced a significant decrease in ROS generation at 4 h, except for CeO\(_2@P3\) at the lowest dose and bare CNPs at 1000 µg mL\(^{-1}\). At 24 h, bare CeO\(_2\) did not show any antioxidant effect. CeO\(_2@P1\) exhibited an antioxidant effect at 100 µg mL\(^{-1}\) (p < 0.05), CeO\(_2@P2\) at both 100 and 1000 µg mL\(^{-1}\) (p < 0.01), and CeO\(_2@P3\) at 1000 µg mL\(^{-1}\) (p < 0.001). Taken together, our data demonstrate that our coatings preserve the antioxidant capacities of CNPs in a model relevant to ischemia. First of all, glutamate significantly increased ROS levels in bEnd.3 cells, which is consistent with previous studies performed on bEnd.3 cells.[66,67] Although numerous studies have shown antioxidant effects of CNPs (reduction in ROS production by 30% to 50% at concentrations of 1 to 200 µg mL\(^{-1}\))[16,24,60] only few of them have been performed on endothelial cells.[32,58,60] Additionally, most studies used H\(_2\)O\(_2\) to induce oxidative stress,[16,24,60] although it may partially react with the probe as well as CNPs in the extracellular medium.[68]

**Effect of Nanoceria on Mitochondrial ROS Production:** We then explored the effect of nanoceria especially on mitochondrial production of superoxide anions using the MitoSOX Red reagent. In a first step, we evaluated the mitochondrial ROS production after a 4 h treatment with glutamate (100 mM) or H\(_2\)O\(_2\) (2 mM) (Supporting Information, S6). The percentage of MitoSOX Red reagent-positive cells was not increased by glutamate compared to control cells (Figure S6, Supporting Information). By contrast, H\(_2\)O\(_2\) significantly increased the number of labeled cells (p < 0.01), in agreement with another study performed on bEnd.3 cells.[68] Based on these results, the effect of CNPs on mitochondrial production of superoxide anions was evaluated in the presence of H\(_2\)O\(_2\) at 2 mM (Figure 7a). Incubation with H\(_2\)O\(_2\) induced a fourfold increase in the fluorescence intensity per cell (112 ± 13 AU, p < 0.001 vs control cells). Bare CNPs did not induce any change in fluorescence intensity per cell (122 ± 13 AU) while it tended to decrease with coated CNPs: a non-significant 27% decrease for CeO\(_2@P1\) (82 ± 15 AU), a 34% reduction close to statistical significance for CeO\(_2@P2\) (74 ± 13 AU, p = 0.0848), and a significant 40%-reduction for CeO\(_2@P3\) (67 ± 6 AU, p < 0.05,
Figure 6. Effect of nanoceria on b.End3 ROS production in oxidative conditions. ROS production was measured with H_2-DCF-DA after 4 and 24 h of incubation. Results were expressed in percentage of fluorescence of control cells. Glutamate (100 mM) was used as pro-oxidant and NAC was used as antioxidant benchmark at 1 mM. Bare nanoceria (a,e), CeO_2@P1 (b,f), CeO_2@P2 (c,g) and CeO_2@P3 (d,h) were incubated at 10, 100, and 1000 µg mL\(^{-1}\). Data are expressed as mean ± SEM, \(n = 8–16\) at 4 h and \(n = 6–14\) at 24 h, \(*p < 0.05\), \(**p < 0.01\), and \(***p < 0.001\) versus control, \(*p < 0.05\) \(**p < 0.01\), and \(***p < 0.001\) versus glutamate.

Figure 7a). Due to the fast degradation of superoxide anions, our study only focused on the 4 h treatment. A study conducted on astrocyte showed increased mitochondrial superoxide anion production minutes after glutamate treatment.[69] Although CNPs induced a 32–47% decrease in mitochondrial superoxide anion production, only CeO_2@P3 showed a statistically significant effect. Another study demonstrated a significant decrease of only 15% in MitoSox Red levels after addition of CNPs in a hippocampal slice ischemia model.[27]

Effect on Total Thiols: Total thiols have a crucial role as an endogenous antioxidant system. Thiol functions (-SH) maintain the redox state of cells and represent one of the first barriers against
ROS which oxidize them, leading to the formation of disulfide bridges (S–S). A decrease in the amount of total thiols is therefore a reflection of oxidative stress. The amount of total thiols on bEnd.3 resting cells was $156 \pm 7 \mu$mol $\mu$g$^{-1}$ of protein and $72 \pm 4 \mu$mol $\mu$g$^{-1}$ of protein respectively after 4 and 24 h (Figure 7b,c). Glutamate treatment drastically reduced the amount of total thiols at both 4 h ($-31\%$, $p < 0.01$) and 24 h ($-68\%$, $P<0.001$). However, none of the CNPs limited the consumption of total thiols induced by glutamate (Figure 7b,c). Only CeO$_2$@P3, after 24 h of treatment, showed a non-significant trend toward a decrease in thiols consumption (+21% compared to glutamate alone; Figure 7c). CNPs were unable to limit the consumption of total thiols. The amount of total thiols was 72 $\mu$mol $\mu$g$^{-1}$ of protein after 4 h and 156 $\mu$mol $\mu$g$^{-1}$ of protein after 24 h (Figure 7b,c).
of total thiols. Other studies that highlighted a reduction of total thiols consumption used higher doses of CNPs: 17.2 mg mL$^{-1}$ in vitro$^{[21]}$ and 60 to 1000 mg kg$^{-1}$ in vivo.$^{[70,71]}$ In addition, these studies used pretreatments with CNPs, from 48 to 72 h before induction of oxidative stress.$^{[21,70]}$ This could suggest a better affinity of ROS for thiols and their consumption even before the CNPs have entered the cells.

**Effect on DNA Damages:** Among the cellular damages related to oxidative stress, we focused on DNA damages. Guanine is the DNA base most susceptible to oxidative damages, the main modification being its oxidation to 8-hydroxyguanine (8-OHdG).$^{[67]}$ We thus investigated the presence 8-OHdG in bEnd.3 cells after treatment with glutamate, whether or not associated with nanoceria. Glutamate induced a statistically significant increase in DNA oxidation of 149% after a 4 h incubation ($p < 0.001$, Figure 7d) and 49% after a 24 h incubation ($p < 0.01$, Figure 7e). At 4 h, bare CeO$_2$, CeO$_2$@P1 and CeO$_2$@P3 induced a significant decrease in glutamate-induced DNA oxidation of 46% ($p < 0.001$), 26% ($p < 0.001$), and 23% ($p < 0.05$) respectively. CeO$_2$@P2 CNPs have no effect on DNA oxidation (Figure 7d). At 24 h, although a non-significant trend was observed for bare CeO$_2$ (−24%; $p = 0.0784$) and CeO$_2$@P3 (−15%). Only CeO$_2$@P1 significantly diminished the glutamate-induced DNA oxidation of 41% ($p < 0.001$; Figure 7e). CeO$_2$@P2 reduced nucleic acid oxidation at both 4 and 24 h and only at 24 h for CeO$_2$P3. These results were consistent with another study using short time increases in 8-OHdG levels following hypoxia, reduced by 23% by pretreatment by CNPs.$^{[72]}$

In conclusion, in vitro experiments have shown that our coatings limit the CNPs’ toxicity without altering either their internalization nor their antioxidant capacities. All coated CNPs substantially display the same overall antioxidant capacities but only CeO$_2$@P3 reduced significantly the production of mitochondrial superoxide anion (Figure 7a) and only CeO$_2$@P1 reduced significantly the DNA oxidation at 24 h (Figure 7e). In addition, CeO$_2$@P3 carries more amine functions than CeO$_2$@P2, those also being more easily accessible, possibly allowing a greater grafting capacity of targeting peptides. CeO$_2$@P3 and CeO$_2$@P1 (used as a control) have thus been selected for further in vivo studies.

### 2.3. In Vivo Study of Nanoceria Toxicity

#### 2.3.1. Biodistribution of Nanoceria

The biodistribution of CeO$_2$@P3-Cy5 (5 mg kg$^{-1}$) was followed over one month by measuring fluorescence on the whole mouse and on specific organs using regions of interest (ROIs) (Figure 8). The front part acquisitions revealed a stable and maximal fluorescence intensity in the first minutes following the CeO$_2$@P3’s injection and significantly higher than CeO$_2$@P1 (66 ± 14, 66 ± 11, and 75 ± 16 AU respectively at 5, 15, and 30 min; $p < 0.001$; Figure 8b). Then, the amount of fluorescence slowly decreased and returned to basal level around 14 days post-injection (0.7 ± 0.2 AU). Concerning the back part acquisitions, the fluorescence intensity was 3.5-fold weaker (18 ± 2 AU at 5 min) and elimination was faster (Figure 8e). The higher fluorescence level in the front of the mice (Figure 8b,e) suggested the presence of CNPs in spleen, liver (Figure 8c), and bladder (Figure 8d) rather than kidneys (data not shown).

The ROI placed on the liver showed a large and stable amount of CNPs for up to 30 min (60 ± 7, 70 ± 8, and 58 ± 5 AU, respectively at 5, 15, and 30 min; $p < 0.001$; Figure 8c), which started to decrease from 60 min post-injection (42 ± 5 AU; $p < 0.001$), this reduction being confirmed in the following hours (12 ± 2 and 8 ± 1 AU respectively at 3 and 24 h). Only the ROI drawn on the bladder showed a very important fluorescence peak at 30 min (257 ± 87 AU; $p < 0.01$; Figure 8d), followed by a rapid decrease to reach a negligible amount from 6 h post injection.

Twenty-four hours after injections (Figure 8f) CeO$_2$@P3-Cy5 were mainly found in the liver (33.2 ± 1.6 µg g$^{-1}$; $p < 0.001$) and spleen (29.8 ± 1.4 µg g$^{-1}$; $p < 0.001$). A small amount was also found in the kidneys (3.3 ± 0.2 µg g$^{-1}$; $p < 0.01$) and lungs (3.1 ± 0.2 µg g$^{-1}$; $p < 0.05$). One month after injections, CeO$_2$@P3-Cy5 were still mainly found in the spleen (10.1 ± 1.4 µg g$^{-1}$; $p < 0.001$) and liver (3.2 ± 0.6 µg g$^{-1}$; $p < 0.001$). A small, non-significant amount of NPCs were found in other organs. In addition, the amount of CeO$_2$@P3-Cy5 in the liver and spleen decreased significantly compared to 24 h (p < 0.001). Taken together, our data demonstrated CeO$_2$@P3-Cy5’s elimination within the first hours after injection. Elimination appeared to be mainly mediated by the liver and spleen with short-term accumulation in these organs. Given the Gaussian size distribution of the CeO$_2$@P3-Cy5 measured by DLS (from 10 to 100 nm; data not shown), the smallest NPCs could pass through the kidneys and then the bladder. Most studies were consistent with our data and detected more CNPs in liver and/or spleen compared to bladder or kidneys.$^{[18,73–78]}$ These studies showed an amount of CNPs in the liver 24 h after the injections from 7$^{[18,75]}$ to 25 µg g$^{-1}$.$^{[74]}$ To note, a study demonstrated a significant concentration of NPC in the bladder in the first hours after the injection without any trace found in the liver and spleen.$^{[79]}$

#### 2.3.2. Toxicity of Nanoceria

Toxicity of CeO$_2$@P3-Cy5 and of CeO$_2$@P1 used as a control was examined in male Swiss mice. Blood count and anatomo-pathological analysis were performed at both 24 h and 1 month after nanoceria (5 mg kg$^{-1}$) or PBS intravenous injection. Despite a slight but significant difference in lymphocytes and neutrophils percentages between CeO$_2$@P3-Cy5-treated and control mice (Table 3, $p < 0.01$), blood count values are within the physiological ranges, which indicates a lack of toxicity of our CNPs in the mouse up to 1 month after a single injection at a dose of 5 mg kg$^{-1}$.$^{[18,80]}$ Our results are in agreement with studies using other CNPs at 0.5 or 5 to 20 mg kg$^{-1}$.$^{[18,80]}$ To note, at higher concentrations (from 30 to 300 mg kg$^{-1}$), it has been reported that CNPs can induce toxicity on blood formula especially on leucocytes and lymphocytes.$^{[76]}$

Concerning anatomo-pathological analysis, regarding the spleen, CNPs did not induce any lesion, and the distribution and the size of follicles was unaffected by the treatment neither at 24 h (data not shown) nor at 1 month post injection (Figure 9). The homogenous distribution of the white and red pulp highlights the representativeness of the cell types and the immunocompetence of the observed spleens. For the liver, no lesion or fibrosis...
Figure 8. Biodistribution of CeO$_2$@P$_3$–Cy$_5$ at 5mg kg$^{-1}$ in mice. a) Evolution of fluorescence intensity on frontal acquisitions at different times on control (C), CeO$_2$@P$_1$ (P1) and CeO$_2$@P$_3$–Cy$_5$ (P3) mice. Cyanine quantifications after acquisitions of b) front, c) liver, d) bladder, and e) back. The fluorescence intensity was expressed in photon/second/cm$^2$/steradian and was calculated from the formula: \[\frac{\text{ROI}_{\text{CNP at } T_X} - \text{ROI}_{\text{CONTROL at } T_X}}{\text{ROI}_{\text{STANDARDCYANINE at } T_X}}\]. Data are expressed as mean $\pm$ SEM ($n=3$ for control and CeO$_2$@P1 and $n=9$ for CeO$_2$@P$_3$–Cy$_5$). Quantification of cyanine in the organs of mice (liver, kidney, spleen, lungs, and brain) f) 24 h and g) 1 month after injection. Data are expressed as mean $\pm$ SEM ($n=6$). 2-way ANOVA and Sidak's test, *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$ versus CeO$_2$@P1 and $$$p < 0.001$ versus 24 h.

was induced by CNPs injections. Some regions of regeneration were observed but no difference was observed compared to control mice. In kidneys, no CNPs-induced lesions were observed, especially in glomeruli and Bowman's capsules. Regarding the lungs, no fibrosis was observed following the injection of CNPs. Some hemorrhagic infiltrates were detected especially in the alveolar ducts. However, these infiltrates were also detected in control mice and would therefore seem to be a post-mortem artefact. Finally, no lesions were observed in the brain after CNPs injection. Most studies reported an absence of CNPs toxicity.
Table 3. Blood count of mice treated with PBS (control), CeO$_2$@P1, and CeO$_2$@P3-Cy5.

|                | Control       | CeO$_2$@P1    | CeO$_2$@P3-Cy5 |
|----------------|---------------|---------------|----------------|
|                | 24 h          | 1 month       |                |
| Leukocytes     | 4.6 ± 0.7     | 4.1 ± 0.5     | 4.5 ± 0.5      |
| Lymphocytes [%]| 69.6 ± 2.1    | 62.3 ± 3.0    | 57.3 ± 1.9**   |
| Monocytes [%]  | 5.9 ± 0.4     | 5.6 ± 0.3     | 5.9 ± 0.4      |
| Neutrophils [%]| 20.0 ± 2.0    | 27.2 ± 2.5    | 31.0 ± 1.9**   |
| Eosinophils [%]| 1.1 ± 0.6     | 1.4 ± 0.3     | 1.7 ± 0.5      |
| Basophils [%]  | 1.7 ± 0.2     | 1.7 ± 0.1     | 1.4 ± 0.3      |
| Erythrocytes   | 8.3 ± 0.3     | 8.4 ± 0.4     | 8.3 ± 0.2      |
| Hematocrit [%] | 41.5 ± 2.1    | 43.1 ± 2.2    | 42.4 ± 1.0     |
| Hemoglobin [g dL$^{-1}$] | 10.6 ± 0.5 | 11.6 ± 0.3 | 11.1 ± 0.3 |
| Thrombocytes   | 1809 ± 93     | 1786 ± 114    | 1719 ± 73      |

Nanoceria were administrated intravenously (5 mg kg$^{-1}$, 5 mL kg$^{-1}$ in PBS 0.1 m) and blood count was assessed 24 h and 1 month after injection. **: $p < 0.01$ versus control.

Figure 9. Anatomo-pathological analysis of mice treated with PBS (control), CeO$_2$@P1 or CeO$_2$@P3-Cy5 one month after injection. PBS and nanoceria were administrated intravenously (5 mg kg$^{-1}$, 5 mL kg$^{-1}$ in PBS 0.1 m).

Similar results have been reported 24 h to 3 months after injection of CNPs at doses of 0.5 to 100 mg kg$^{-1}$.[18,74,75] The only studies that demonstrated toxicity after injection of CNPs used very high doses: greater than 250 mg kg$^{-1}$ by intravenous injection[76] and 641 mg m$^{-3}$ by inhalation[81]. Overall, these data demonstrate the lack of toxicity of CNPs both 24 h and 1 month after a single injection of coated CNPs at 5 mg kg$^{-1}$. No abnormal blood count, lesion, necrosis, fibrosis, cellular regeneration, significant hemorrhagic, or immune infiltrate was observed. The lack of in vivo toxicity of our coated CNPs...
allows us to plan the next study of their antioxidant effect in an experimental model of cerebral ischemia to assess their potential for therapeutic use.

3. Conclusion

To conclude, we formulated CNPs coated with statistical multiphosphonate-PEG copolymers as enzyme-mimicking catalysts for the decomposition of ROS. The polymers examined have a dual functionality, one for protection against protein adsorption in the form of a PEGylated corona and one for targeting thanks to terminal amine groups allowing further covalent binding. These polymers impart remarkable colloidal stability to metal oxide nanoparticles in biological media. Keeping the CNP core identical and changing the nature of the coating, we studied the impact of polymers on the CAT- and SOD-like catalytic activities of bare and coated cerium oxide. We observed that the multi-PEG coatings did not affect the SOD-like and slightly impair the CAT-like effect of nanoceria, a result that confirms the benefit of having phosphonic acids as anchoring groups at the particle surface. In vitro assays performed on murine cerebral endothelial cells showed that these coated CNPs do not exert any toxicity while interacting with cells, as demonstrated by ICP-OES and flow cytometry measurements. Confocal microscopy and TEM studies notably indicated a perinuclear cytoplasmic CNP localization, more precisely in the endosomes. Coated CNPs were able to reduce intracellular glutamate-induced ROS production, showing that the coating does not decrease the antioxidant effect. While all coated CNPs exert substantially the same antioxidant effects, evaluation of mitochondrial ROS production and DNA oxidative damages allow identifying CeO$_2@$P3 as the most effective nanoparticles. Finally, IV administration of CeO$_2@$P3 in mice highlighted a lack of toxicity on blood count and anatomico-pathological analysis up to 1 month as well as an elimination mainly via the liver and the spleen. The present work therefore enables us to identify biocompatible CNPs with very interesting antioxidant effects that can be used for the treatment of cerebral ischemia. The continuation of this work will therefore consist in evaluating their efficacy in models of stroke, for subsequent clinical application. It will also be of particular interest to investigate whether the grafting of targeting agents onto these particles would make it possible to concentrate their antioxidant effects in the affected areas, thus increasing their beneficial effects.

4. Experimental Section

Materials: The CNPs aqueous dispersion (200 g L$^{-1}$, pH 1.5) was synthesized by Rhodia (Centre de Recherche d’Aubervilliers, Aubervilliers, France) [49,50]. Polymers were synthesized and provided by SPECIFIC POLYMERS (Castries, France). PEG mono-functional vinyl ether (Vinyl-PEG, CAS: 79−41−4) was supplied by Ines and used as received. Methacryloyl chloride (CAS: 920−46−7, Sigma Aldrich) was distilled before use. Dimethyl(methacryloyloxy)methyl phosphonate (MPh$_2$, SP-41-003, CAS: 86242−61−7) monomers were produced by SPECIFIC POLYMERS. Cysteamine hydrochloride (CAS: 156−57−0), di-tert-butyl dicarbonate (Boc$_2$O, CAS: 24424−99−5), 2,2′-azobis(isobutyronitrile) (AIBN; CAS: 78−67−1) were supplied by Sigma Aldrich and used as received.

Transmission Electron Microscopy: Micrographs were taken with a Tecnai 12 TEM operating at 80 kV equipped with a 1K x 1K View camera. Nanoceria dispersions were deposited on ultrathin carbon type-A 400 mesh copper grids (Ted Pella, Inc.). Micrographs were analyzed using Image software for 200 particles.

X-Ray Photoelectron Spectroscopy: XPS data were collected using an Omicron Argus X-ray photoelectron spectrometer using a monochromatic Al$_{K\alpha}$ (1486.6 eV) radiation source with a 300 W electron beam power. The emission of photoelectrons from the sample was analyzed at a takeoff angle of 45° under ultra-high vacuum conditions (10$^{-8}$ Pa). The spectra were collected at pass energy of 100 eV for the survey scan and 20 eV for the high-resolution scans. The XPS spectra were fitted using the software XPS-Peak41, applying a fixed Gaussian/Lorentzian ratio for peaks of the same spectrum and constraining the full-width at half-maximum of each doublet to be equal. The fraction of Ce$^{3+}$ was determined through the ratio between the integrated intensities of the four peaks corresponding to Ce$^{3+}$ divided by the integrated intensities of all ten peak.

Coating Cerium Oxide Nanoparticles: CNPs were coated with phosphonic PEG copolymers using a formulation pathway described earlier [48,51]. In brief, particle and polymers dispersions were prepared in the same conditions of pH (pH 1.5) and concentration (c = 2 g L$^{-1}$) and mixed at different volume ratios. The CNP dispersion was added dropwise to the polymer solution under magnetic stirring keeping the mixing volume ratio at X$_C/N$s where X$_C$ denotes the critical mixing ratio (nanoparticle over polymer) above which the CNPs are partially coated and precipitate at physiological pH [49]. Working at X$_C/N$s insured that polymers were in excess during adsorption. After increasing their pH to 8 by addition of NaOH, the dispersions were centrifuged at 4000 rpm using Merck centrifuge filters (pore 100 000 g mol$^{-1}$) to remove the polymer excess and further concentrated to 20 g L$^{-1}$. The hydrodynamic diameter D$_H$, electrophoretic mobility and zeta potential were obtained using DLS and electrophoretic measurements (NanoZS Zetasizer spectrometer, Malvern Instruments). The hydrodynamic diameters provided here are the second coefficients in the cumulant analysis derived from the Stokes–Einstein relation D$_H$ = k$_B$ T/3πηD$_C$ where k$_B$ is the Boltzmann constant, T the temperature, η the solvent viscosity and D$_C$ the average diffusion coefficient. Measurement were performed in triplicate at 25 ºC after an equilibration time of 120 s. A UV–vis spectrometer (SmartSpecPlus from BioRad) was used to measure the absorbance of polymer coated nanoceria aqueous dispersions. Absorbance data were used to determine the nanoparticle concentration for each batch by means of Beer–Lambert law.

SOD Mimetick Activity Assay: The SOD kit assay was purchased from Sigma Aldrich (Lyon, France). The catalytic activity of nanoceria in the dismutation of superoxide radical anion was assessed by a colorimetric assay using UV–vis spectroscopy (Kit #19160-1KTF). Briefly, 20 µL of a nanoceria dispersion in Tris-Cl buffer pH 7.5 was added to a well of a 96-well plate and mixed with 200 µL of WST-1 (2-(4-Iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt). The reaction was initiated with the addition of 20 µL of xanthine oxidase solution, prepared by mixing 5 µL of the enzyme in 2.5 mL of adilution buffer provided. After incubation at 37 ºC for 30 min, the absorbance at 450 nm was measured using a microplate reader (EnSpire Multimode Plate Reader, Perkin Elmer). Nanoceria final concentration ranged from 1 to 1000 µg mL$^{-1}$.

CAT Mimetic Activity Assay: The Amplex Red CAT assay kit was obtained from thermo Scientific (Illkirch, France). The catalytic activity of nanoceria in the disproportionation of H$_2$O$_2$ was assessed by spectrofluorimetry using the Amplex-Red reagent assay (Cat # A22180). Briefly, 25 µL of each nanoceria dispersion in Tris-Cl buffer pH 7.5 was added to a well of a 96-well plate and mixed with 25 µL of a H$_2$O$_2$ solution, to obtain an H$_2$O$_2$ concentration of 5 µm in each well. Then, 50 µL Amplex Red reagent/chorseraldich peroxide (HRP) working solution was added and reactions pre-incubated for 5 min. Amplex Red (10-acetyl-3,7-dihydroxynaphoxazene) reaction with H$_2$O$_2$ catalyzed by HRP produces the fluorescent molecule resorufin (excitation at 571 nm and emission at 585 nm). The fluorescence was measured after incubating for 30 min with protection from light. The final H$_2$O$_2$ concentration in each well was 5 µm, whereas those of nanoceria ranged from 1 to 1000 µg mL$^{-1}$.

Cell Culture: Immortalized mouse brain endothelial cells, bEnd.3 (ATCC CRL-2299, Manassas, Virginia, USA) purchased from Sigma (Sigma Aldrich, Saint Quentin Fallavier, France) were cultivated in Dulbecco’s modified Eagle’s medium (DMEM) Glutamax (Gibco, 31966-021),
supplemented with 10% foetal bovine serum (FBS, Dutscher S1810-500, 100 µM L−1 penicillin and 100 µg mL−1 streptomycin (Gibco, 15140122) in a humidified 5% CO₂ incubator at 37 °C. Cell line passages < 30 were used for all experiments. To evaluate mortality, metabolic activity and measure ROS, bEnd.3 cells were seeded in 96 well plates at 5 × 10^4 cells per well (≈ 100 000 cells cm⁻²); for mitochondrial ROS detection, cells were seeded into 24-well plates at a density of 200 000 cells per well (≈ 100 000 cells cm⁻²). Twenty-four hours after, treatments were applied (Glutamate 100 mM, NAC 1 mM, H₂O₂ 2 mM, and CNPs at 10, 100, and 1000 µg mL⁻¹) diluted in DMEM without FBS and without phenol red (Gibco, 21063-09), and incubated with cells during 4 or 24 h.

**Trypan Blue Count:** The cells were detached from the wells by trypsin-EDTA (0.25%), inactivated by complete medium, and centrifuged at 100 g for 5 min. Cells were then taken up in 100 µL of PBS with 1% of bovine serum albumin (BSA) and stained with trypan blue 0.2% (Sigma, T8154). Blue and white cells were then counted in Kova slides (Dutscher, 050126) with a minimum of 150 cells. The percentage of mortality was calculated: 100 × (blue cells / (white + blue cells)).

**MTT Assay:** A solution of 5 mg mL⁻¹ of 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT, Sigma, S12045) was prepared, and 10 µL of each was added to well (final concentration at 0.5 mg mL⁻¹) and incubated for 3 h. MTT was reduced to formazan by mitochondrial succinate dehydrogenase within viable cells. The wells were emptied, and formazan was dissolved in DMSO (23.488.294, Prolabo, Fontenay-sous-Bois, France). Optical Density was read at a wavelength of 570 nm and nonspecific absorbance at 690 nm was removed. Results were expressed in percentage of absorbance of control cells.

**Inductively Coupled Plasma-Optical Emission Spectrometry:** Two million bEnd.3 cells (2 × 10⁵) were incubated into 6-well plates (≈ 100 000 cells cm⁻²) for 24 h with CNPs dispersed in DMEM (1, 10, 100, or 1000 µg mL⁻¹), combined or not with glutamate 100 mM. Cells were washed with PBS and digested with concentrated nitric acid (HNO₃, Prolabo, 20.425.297) and hydrogen peroxide (10 µL). After evaporation the dry samples were diluted with HNO₃ 2%. For cerium detection, ICP-OES experiments were performed at the Institut Physique du Globe de Paris (IPGP) using an iCAP6200 ThermoFisher spectrometer. The measurements were performed in triplicate at radiation wavelengths 393.11 and 404.08 Å with an uncertainty better than 5%.

**Cyanine Labeling:** The primary amine groups of CeO₂@P2 and CeO₂@P₃ were quantified by spectrofluorometry (Supporting Information 4). CeO₂@P2 and CeO₂@P₃ were labeled with Cyanine 5 (Lumiprobe, #23320) in PBS buffer adjusted to pH 8 (2 h at room temperature while stirring continuously). Particles were washed five times with PBS on an exclusion column (30 kDa) to remove the free Cyanine. The same experiment was performed in triplicate at radiation wavelengths 580 nm and an emission wavelength of 535 nm.

**Quantification of Total Thiols:** After treatments, bEnd.3 cells were washed by PBS. Three freezings-thawings cycles were carried out (−80 to 37 °C). Standard of reduced glutathione was performed from 0 to 50 µM. Thioli Green Indicator 2X solution (156049, Abcam, Paris, France) was prepared and diluted at 1:4 and incubated for 20 min in each well. Fluorescence intensity was measured with a Tecan fluorimeter (Infinite F200 Pro) at an excitation wavelength of 485 nm and an emission wavelength of 535 nm.

**Confocal Fluorescence Microscopy:** For microscopy studies, the cells were cultured in 24 well plates on coverslips (Kittel Glass, Brunswick, Allemagne) at a density of 200 000 cells per well in DMEM medium and cultured for 24 h as described above. The cells were treated with DMEM (control cells), glutamate (100 mM) alone or associated with CNPs (CeO₂@P1, CeO₂@P₂-Cy₅, and CeO₂@P₃-Cy₅) at 100 µg mL⁻¹ in DMEM medium free of FBS and phenol red (Gibco, 21063-09) for 4 h. Cells were washed with PBS, fixed with 4% (w/v) paraformaldehyde (16965002S, ACROS, Fisher Scientific) for 12 min. Non-specific sites were blocked with PBS–goat serum 5% (Abcam, Cambridge, MA) for 30 min. Mouse anti-8-OHdG antibody (SantaCruz, sc66036 Clone 15A3, 1/100) was incubated overnight at 4 °C. The AlexaFluor anti-mouse A555 (Invitrogen, A10003) was incubated (1/500) 2 h at room temperature. DAPI was incubated for 5 min (0.1 µg mL⁻¹ in PBS, Merck Millipore, 268298). The slides were mounted with mowiol (Mowiol Sigma 81381, Mowiol Sigma Guava Easy Cyte Ds200) at excitation wavelengths of 485 nm and emission wavelengths of 535 nm.

**Quantification of DNA Damages (8-OHdG):** DNA oxidative damages were studied by investigating the presence of 8-OHdG in bEnd.3 cells. Cells were seeded in 24 well plates on coverslips (Kittel Glass, Brunswick, Allemagne) at a density of 200 000 cells per well in DMEM medium and cultured for 24 h. Cells were treated by CeO₂@P1 and CeO₂@P₂ at 1000 µg mL⁻¹ in DMEM medium without FBS and without phenol red for 24 h. Cells were washed with PBS and fixed with 3% glutaraldehyde and 2.5% PFA at 4 °C for 48 h. Cells were scraped and included in 2% gelatin. The pellets were recovered and centrifuged at 250 g for 1 min, followed by a second fixation with 1% osmium tetroxide (OsO₄) and potassium ferrate (FeK) for 1 h at room temperature. An inclusion in 1% agarose was performed and cut into cubes. The dehydration was carried out by successive ethanol baths: 30% (15 min); 50% (15 min), 70% (15 min), 90% (15 min) 100% (2 × 15 min), and 100% ethanol with propylene oxide (2 × 15 min). The impregnation was performed in propylene oxide/epoxy resin (Epon) at ratio 1:1 overnight followed by 2 baths of Epon for 1 h. The inclusion was followed by polymerization at 60 °C for 18 h. TEM was performed on 70 nm thick microtome cell sections. The micrographs were made on a Tecnai 12 TEM of 80 kV equipped with a camera 1K × 1K KeenView.

**Flow Cytometry:** Adsorption/internalization was evaluated by flow cytometry on bEnd.3 cells cultured in 24 well plates (2 × 10⁵ cells per well ≈ 100 000 cells cm⁻²) in DMEM Glutamax during 24 h. Cyanine 5 labeled CeO₂@P₂ and CeO₂@P₃ (100 µg mL⁻¹) in DMEM without FBS and without phenol red (Gibco, 21063-09) were incubated during 4 or 24 h. Cells were washed 3 times with PBS, detached from the wells by trypsin, inactivated by complete medium without phenol red, centrifuged for 5 min at 100 g and the supernatant was removed. The MitoSOX Red reagent (Thermofisher, M36008) diluted in PBS (5 µM) was added in each tube with PBS–BSA 1 g L⁻¹ (ID Bio, 1000-70)–EDTA (200µM, Sigma, ED4555) at ratio 1:4 and incubated for 20 min. Cellular uptake was quantified with FACs (Millipore Guava Easy Cyte Ds200) at excitation wavelengths of 485 nm and emission wavelengths of 535 nm.

**Mitochondial ROS Detection:** The MitoSOX Red reagent is a compound that penetrates living cells and selectively targets mitochondria. After incubation of the different treatments, the cells were washed three times with PBS and detached from the wells by trypsin, inactivated by complete medium without phenol red, centrifuged for 5 min at 100 g and the supernatant was removed. The MitoSOX Red reagent (Thermofisher, M36008) diluted in PBS (5 µM) was added in each tube with PBS–BSA 1 g L⁻¹ (ID Bio, 1000-70)–EDTA (200µM, Sigma, ED4555) at ratio 1:4 and incubated for 20 min. Cellular uptake was quantified with FACs (Millipore Guava Easy Cyte Ds200) at excitation wavelengths of 485 nm and emission wavelengths of 535 nm.
images were taken with an epifluorescence microscope (Zeiss Axiopt, Marly le Roi, France) at a wavelength of 550 nm. The analyses were carried out using Image J software. Fluorescence intensity was quantified at the nucleus level using DAPI labeling.

**In Vivo Study:** All experiments were carried out in accordance with the European Parliament Directive of September 22, 2010 (2010/63/EU) and the French regulations regarding the protection of animals used for experimental and other scientific purposes (D2013-118), with the approval of both the Paris Descartes University Animal Ethics Committee (Comité d’Éthique pour l’expérimentation animale de Paris Descartes, CEEA 34) and the Ministry of Education and Research (registered number APAFIS Project #13638).

Six-week-old male Swiss mice (28–32 g, Janvier Labs, Le Genest-Saint-Ise, France) were housed under standard conditions with a 12 h light/dark cycle and allowed access to food and water ad libitum. Mice were injected i.v. with the air-oxygen mixture and placed in a Photon Imager (Biospace Lab, Nesles-la-Vallée, France). Acquisitions at early times were carried out for the analysis of cyanine fluorescence intensity from ROI in different organs at 24 h and 1 month after the injections. Organs were washed, weighed, and processed for histology using 5 µL of 0.5 m EDTA (Sigma, 03660) and used for blood count (NFS, MS95, Melet Schloesing Laboratories, Osny, France).

Immediately after, animals were decapitated, the organs were removed and post-fixed with paraformaldehyde 4% (Euromedex, 16%, 15700, Soufflewyersheim, France). PBS/0.1% triton volume was added to the samples to dehydrate and clarify the samples using 70% and 100% alcohol baths and with isopropanol and then incubated in xylool, and mounted with DPX (List, Rosenfeld, Germany).

Toxicity: An intracardiac blood sample (1 mL) was collected in a heparinized syringe, and 5 and 97 mL tubes containing ceramic beads (P000918 and P000935, Precellys Lysing Kit, Ozyme, Saint-Cyr-l’ École, France). Organs were mechanically ground by two cycles 6000 rpm during 30 s in PBS/Triton 0.1% (02520-300R-D000, Precellys evolution, Bertin Technologie, Montigny-le-Bretonneux, France). PBS/0.1% triton volume was used to wash the organs and to adjust the weight of each organ. Lysate were transferred to 5 and 7 mL tubes containing ceramic beads (P000918 and P000935, Precellys Lysing Kit, Ozyme, Saint-Cyr-l’ École, France). Organs were homogenized at 10 000 g for 10 min. Cyanine fluorescence was measured with a Tecan fluorimeter (Infinite F200 Pro) with an excitation wavelength of 620 nm and emission wavelengths of 670 nm. Lysate were centrifuged at 10 000 g for 10 min. Cyanine fluorescence was measured with a Tecan fluorimeter (Infinite F200 Pro) with an excitation wavelength of 620 nm and emission wavelengths of 670 nm.

**Biodistribution:** Animals were anesthetized with isoflurane (2% in an air-oxygen mixture) and placed in a Photon Imager (Biospace Lab, Nesles-la-Vallée, France). Acquisitions at early times were carried out for the analysis of cyanine fluorescence intensity from ROI in different organs at 24 h and 1 month after the injections. Organs were washed, weighed, and transferred to 5 and 7 mL tubes containing ceramic beads (P000918 and P000935, Precellys Lysing Kit, Ozyme, Saint-Cyr-l’ École, France). Organs were homogenized at 10 000 g for 10 min. Cyanine fluorescence was measured with a Tecan fluorimeter (Infinite F200 Pro) with an excitation wavelength of 620 nm and emission wavelengths of 670 nm.

**Quantification of cyanine:** Cyanine was carried out in different organs at 24 h and 1 month after the injections. Organs were thawed, weighed, and transferred to 5 and 7 mL tubes containing ceramic beads (P000918 and P000935, Precellys Lysing Kit, Ozyme, Saint-Cyr-l’ École, France). The samples were centrifuged at 10 000 g for 10 min. Cyanine fluorescence was measured with a Tecan fluorimeter (Infinite F200 Pro) with an excitation wavelength of 620 nm and emission wavelengths of 670 nm.

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**Supporting Information**

Supporting Information is available from the Wiley Online Library or from the author.

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