# Cerium Oxide Nanoparticles: Advances in Biodistribution, Toxicity and Preclinical Exploration

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**Abstract.**

Antioxidant nanoparticles have gained recently tremendous attention for their enormous potential in biomedicine. However, discrepant reports of either medical benefits or toxicity, and lack of reproducibility of many studies, generate uncertainties delaying their effective implementation. In this review, we consider the case of cerium oxide, a well-known catalyst in the petrochemistry industry and one of the first antioxidant nanoparticle proposed for medicine. Like other nanoparticles, it is now described as a promising therapeutic alternative, now as threatening to health. Sources of these discrepancies and how this analysis helps to overcome contradictions found for other nanoparticles are summarized and discussed. For the context of this analysis, we review what has been reported in the liver, where many diseases are related to oxidative stress. Since well-dispersed nanoparticles passively accumulate in liver, it represents a major testing field for the study of new nanomedicines and their clinical translation. Even more, many contradictory works have reported in liver either cerium oxide associated toxicity or protection against oxidative stress and inflammation. Based on this, finally, the intention is to propose solutions to design improved nanoparticles that will work more precisely in medicine and safely in society.

## 1. Introduction

The last three decades have witnessed the emergence of nanotechnology as a "disruptive technology", with great potential to contribute to improved treatments by the generation of new diagnostic and therapeutic products. In particular, inorganic nanoparticles (NPs) have emerged as flexible platforms to develop new imaging and therapy agents for detecting and treating diseases at its earliest stages, with benefits superior to any currently used treatments.[1] These materials have been reported as robust drug carriers, versatile scaffolds able to adjust conjugated biomolecules activity and antennas that can be excited in biologically transparent media.[2] Besides, they can be easily detected and tracked in physiological environments due to their unique physicochemical signatures.[3] Thus, nowadays, with the requirements for more personalized treatments and precision medications,[4] the interest in these materials to develop multimodal/multifunctional nanosized particles that can perform diagnosis[5] and different therapies (such as chemo-, thermo-, radio-, immuno- therapies) in a single nanoplatform[6] is continuously growing.[7]

Amongst the broad range of newly proposed nanomaterials, antioxidant NPs add to this list of advantages that they even show therapeutic action by themselves.[8] Back in 2004, Manea et al. introduced the concept of nanozymes to describe the RNase-like behavior of AuNPs used as catalysts for the cleavage of phosphate esters.[9] From this, nowadays the vast majority of NPs intended for medical applications are inorganic metal (e.g. AuNPs) and metal oxides (e.g. $CeO_2$,[10] $TiO_2$,[11] $Fe_3O_4$,[12] and $MnO_2$[13]). Recently, metal-free NPs (mainly black-phosphorous nanosheets) have been also reported.[14] Those inorganic NPs can be powerful antioxidant and anti-inflammatory agents[15] and they can initiate biological responses to enable different therapies such as photodynamic therapy,[11, 13] chemodynamic therapy[16] and sonodynamic therapy,[17] among others. In addition, they can modulate biological microenvironments for generating therapeutic effects,[18] and thus, they have been proposed

for improving cancer therapy.[19] All this has opened the way for what has been called *"nanocatalytic medicine"*,[20] or *"antioxidant nanomedicine"*.[21]

The recent works of Liu et al.[22] on "antioxidant nanomaterials" and Wang et al.[23] and Ghorbani et al.[24] on "nanozymes" offers a comprehensive review of different types of NPs proposed in the scientific literature. Furthermore, the rationale behind their important role of NPs in nanomedicine, the new developments in this promising therapeutic strategy, and the mechanisms of action of antioxidant nanosystems have been also recently described and reviewed.[20b, 21] Similarly, recent advances in specific enzymatic activities of different nanomaterials have been reviewed such as glucose oxidase[25] and peroxidase activities.[26]

However, and as it happens with other new materials, despite their biomedical potential, little progress is achieved towards translation to clinical practice due to economic, societal and technical aspects. Amongst the latter, plenty of discrepant reports, either showing NPs as promising therapeutic alternatives in medicine or as threatening to health, are still fueling the debate of their safe use.[27] As an example, the same year two different reviews appear pointing out the beneficial[10] and the adverse[28] medical effects of $CeO_2$NPs. In addition, their evolution in physiological environments and their potential toxicity and fate in the long-term are not completely understood.

As a consequence, only very few NPs -few iron oxides- have been approved by regulatory agencies, and only for applications such as iron replacement therapy for the treatment of anemia or as contrast agent for magnetic resonance imaging.[29] In this context, herein, we aim to review first the paradigmatic case of the reactivity of antioxidant cerium oxide NPs ($CeO_2$NPs) in the liver (section 3). Learnings from this case can be extended to other NPs, organs, and tissues, which will help to overcome contradictions and provide solutions to

enable the use of NPs in medicine (sections 4 and 5). $CeO_2$ is selected here as a representative antioxidant NP in medical applications. Being a widely known and used catalyst in the petrochemical industry, it was one of the first NP proposed to be used as a therapeutic agent.[30] Currently, a large number of reports praise its wide spectrum enzyme-mimetic activities and immunomodulatory properties that protect tissues against reactive oxygen species (ROS) overproduction and inflammation **(Figure 1A)**. Hence, $CeO_2$NPs have been shown to modulate oxidative stress in diseases such as retinal degeneration,[31] neurological disorders,[32] ischemia,[33] cardiopathies,[34] diabetes,[35] gastrointestinal inflammation,[36] liver diseases[37] and cancer[38], as well as in regenerative medicine[39] and tissue engineering.[40] Even more, $CeO_2$NPs was the first material tested as antioxidant NP in the space. In 2017, a team of the European Space Agency flown with $CeO_2$NPs and proved that the particles remained stable and provided protection to the muscle cells.[41] In 2019, another experiment started in the International Space Station to test the $CeO_2$NPs activity under conditions of microgravity, to counteract the detrimental effects of microgravity-induced oxidative stress.[41]

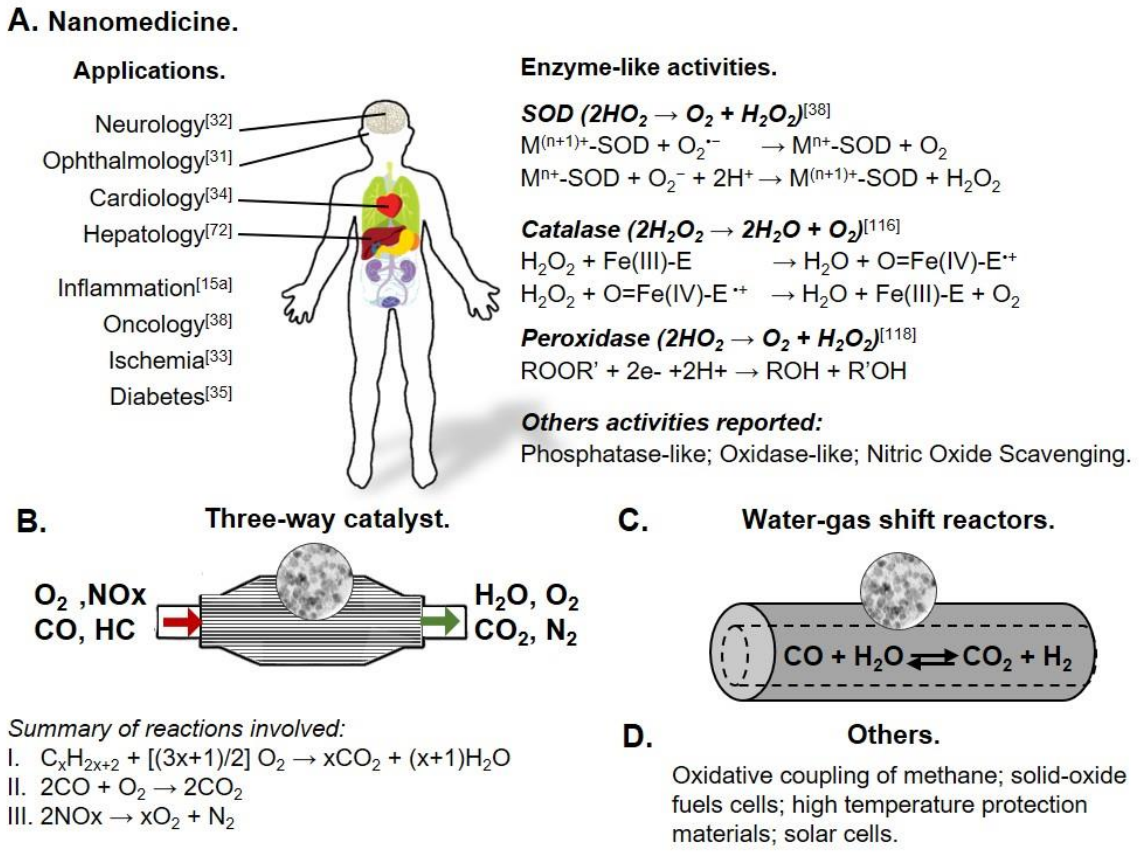


***Figure 1. Different reactions and applications in which $CeO_2$NPs are being proposed or used as antioxidant NPs. A)*** *In nanomedicine research. References are, to the best of our knowledge, the first study reporting the application. We apologize in advance if other contributions were before the ones here listed. Abbreviations: SOD: M=Cu (n=1); Mn (n=2); Fe(n=2); and Ni (n=2); Catalase: Fe(III)-E (heme group iron center attached to catalase; Fe(IV)-E$^{\bullet+}$ (mesomeric form of Fe(V)-E, i.e.,iron not completely oxidized to +V); Peroxidase: The electron donor is very dependent on the structure of the peroxidase. They also may contain in their active site, among others, a heme cofactor or redox-active cysteine or selenocysteine residues.* ***B)*** *In three ways catalytic converters, where I. Oxidation of unburnt hydrocarbons; II. Oxidation of Carbon Monoxide (CO); III. Reduction of Nitrogen Oxides to Nitrogen;* ***C)*** *In water shift reactions and* ***D)*** *Other applications where $CeO_2$NPs are used.*

Along with these interesting applications, the focus on the effects in the liver is a logical approach. First, because it is the organ where the majority of the administered nanomaterials passively accumulate. Thus, it represents a major testing field to start the studies of the NPs evolution, pharmacokinetics, and activity,[42] and consequently, enable the clinical translation of newly developed nanomedicines. Second, the liver is where many discrepant reports show protective effects of $CeO_2$NPs against ROS overproduction and inflammatory processes or the opposite, a role in promoting oxidative stress and toxicity (section 3). These contradictions between therapeutic and deleterious effects have been observed for many other NPs (section

5). Thus, it is reasonable to anticipate that knowledge gain in the liver will pave the way for NPs applications (and other NPs in general) in other organs and tissues once targeted therapies will be a widespread reality.

## 2. $CeO_2$NPs in biomedicine: A historical perspective

The ability of $CeO_2$NPs to balance redox homeostasis in pathological conditions makes it one of the most promising materials to develop new treatments for many diseases. Despite the catalytic capacities of $CeO_2$ are known since long, its powerful medical potential has been evaluated only during the last years, after the pioneering observations of Professor Beverly Rzigalinski and co-workers.[30, 43] Back in 2003, Prof. Rzigalinski and her Ph.D. student David Bailey, at the Virginia State University, unexpectedly observed that $CeO_2$NPs of less than 20 nm prolonged the lifespan of brain cell cultures, for periods of up to 6-8 months.[30c] This finding was described by Prof. Rzigalinski as "somewhat serendipitous" since they were carrying out research using $CeO_2$NPs as a drug carrier.[44]

Thus, the discovery of the pharmacological potential of $CeO_2$NPs is a recent event. It occurred just at the beginning of this century. But, of course, "an unprepared *time* cannot see the outstretched hand of opportunity". This discovery benefited from a long sequence of previous research efforts and results that provided the framework for considering its importance and enabling its continuation as a subject of research **(Figure 2)**. The rare earth (the fifteen lanthanides (Ln), as well as scandium and yttrium) have been found to have biomedical applications since the XIX century. The first one was the use of Cerium Oxalate as antiemetic, of particular use in the sickness that accompanies pregnancy.[45] Subsequently, it came to be prescribed for other gastrointestinal disorders and even for coughs. With the progress of biochemistry knowledge and techniques, medical interest in Ln focused around

the possibilities arising from their ionic radii similar to calcium ions ($Ca^{2+}$) but with higher charge.[46] $Ln^{3+}$ ions were found to have a high affinity for $Ca^{2+}$ sites on biological molecules and rapidly were applied in lowering blood pressure, serum cholesterol and glucose levels, in reducing appetite, as blood coagulation inhibitors and to prevent atherosclerosis in experimental animals.[47]

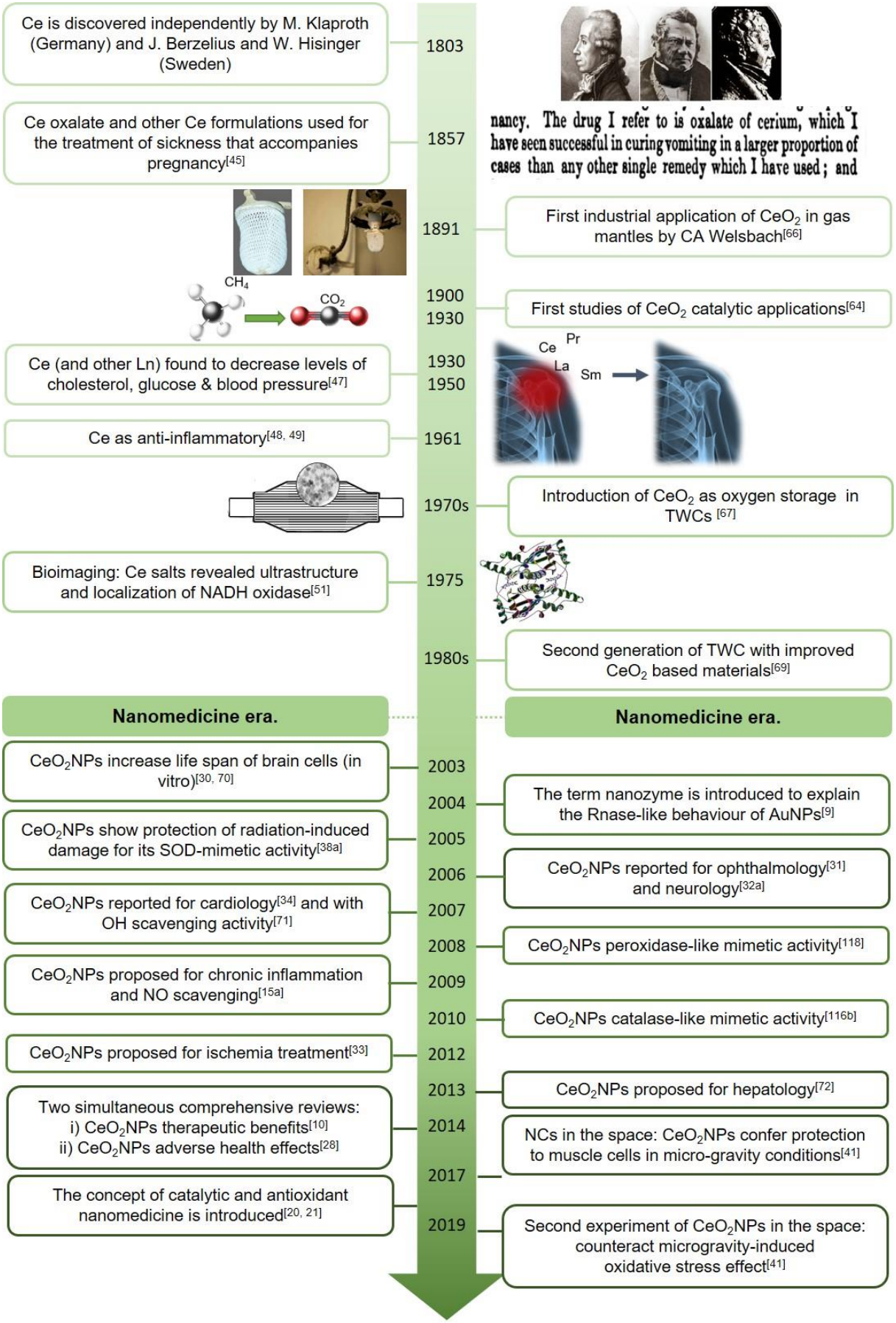


**Figure 2.** Timeline of different achievements using Ce based materials since Ce discovery in 1803.

It was for this anticoagulant role, and following the hypothesis that blood coagulation and inflammation were closely linked processes, that Prof. N. Jancsó introduced the potential anti-inflammatory properties of a variety of Ln.[48] In experiments of late 1950's and early 1960's, he proved in rats that rare earth metals such as $La^{3+}$, $Ce^{3+}$, $Nd^{3+}$, $Pr^{3+}$, and $Sm^{3+}$ were effective, even in the form of their inorganic salts, in inhibiting the angiotaxis and edema that follows the increase in vascular permeability caused by inflammatory agents such as bee venom, cobra venom or dextran.[48] These anti-inflammatory properties were replicated afterward.[49] However, due to excessive toxicity of the Ln salts used (nitrates and chlorides), and the unknown mechanism, Ln did not fulfill their early promise as medically useful anti-inflammatory agents until the recent advent of $CeO_2$ in its nanoparticulate form.

Nevertheless, beyond therapy, several Ln, and particularly Ce, found a successful biomedical use as contrast agents to image specific organs and tissues. A variety of light and electron microscopical histochemistry methods have been developed with the aid of cerium preparations.[50] Again, the bulk of this work has been done with cerium nitrates and chlorides. Briggs et al.[51] introduced the use of cerium chloride for the detection of Hydrogen Peroxide ($H_2O_2$) production to determine the ultrastructural localization of NADH oxidase. This protein was being studied at that time as an enzyme possibly involved in the increased oxidative activity of polymorphonuclear leukocytes (PMN) during phagocytosis. Since then, $Ce^{3+}$, for its electron density and its ability to capture $H_2O_2$ as product of oxidase activity has been profusely used in different techniques using light and electron microscopies for the in-situ detection of the activity of many other oxidases[52] and phosphatases.[53] In these methods, the final reaction products are fine insoluble $Ce^{4+}$-containing precipitates, Ce perhydroxides or Ce phosphates, some in the form of unintended spontaneous NPs, that enable a very precise localization due to their strong reflectance properties. With such techniques, important

advances have been made in cell biology, such as the explanation of extracellular ATPases function and the discovery of new organelles.[50]

In this context, and for this historical perspective, it is worth highlighting the work of Telek et al. in 1999.[54] These authors used a Ce based technique for the *in vivo* histological detection of oxygen-derived free radicals in inflammatory conditions, by quantifying cerium reflectance signals in PMNs. They showed that using DPI (diphenylene-iodonium chloride, a NADPH oxidase inhibitor), SOD (superoxide dismutase) and catalase, the formation of reflectant precipitates around PMNs decreased, confirming their inhibitory action on oxidative stress. However, they observed that SOD also reduced the formation of cerium precipitates. They discussed that one may expect SOD to increase these precipitates since SOD catalyzes the dismutation of superoxide anions to $H_2O_2$ and is $H_2O_2$ which produces the Ce-perhydroxide precipitate. But the opposite was observed. Although potential mechanisms of interactions of Ce with those enzymes were not elucidated, this report hinted a possible role of Ce precipitates in the decrease of the free radical species.

Interestingly, this study was concomitant with the huge rise in the popularity of antioxidants in the 1990s. The role of free radicals and antioxidants in biology was already known since the mid-XX century (see f.i. the works on aging and free radicals by Denham Harman,[55] Linus Pauling works -and philosophy- such as his book *Vitamin C and the Common Cold*[56] and the article published in 1971,[57] or *orthomolecular medicine for everyone* by A. Hoffer and A. W. Saul in 2008).[58] But it was in 1993 when antioxidants attracted attention worldwide as a consequence of a large human study (87,000 female nurses) published in the New England Journal of Medicine. The results of this study suggested that vitamin E supplements could be associated with a reduced risk of coronary heart disease in women.[59] Afterward, other works also reported beneficial effects of antioxidant substances in chronic

inflammatory diseases, neurodegenerative diseases, and cancer, among others.[60] However, other studies pointed out the pitfalls of their use.[61] For instance, only one year after this work, another study showed that the supplementation with Vitamin E and Beta-Carotene did not prevent smoking-induced lung cancer. On the contrary, these supplements could have harmful effects.[62] This study had an 18-year postintervention follow-up with similar results.[63] In fact, since decades ago, it has been recurrently observed how promising preclinical studies of antioxidant therapies failed when translated to the clinic. This has been attributed to the non-druglikeness of available antioxidant compounds. These have high unspecific reactivity and limited absorption profiles, hence low bioavailability and low concentrations at the target site. In this context, radically new antioxidant substances like $CeO_2$NPs, with their ROS buffering capacities and mild but permanent activity, may overcome previous limitations and enable antioxidant therapies to improve human health. This is discussed in more detail in section 4.

In parallel to this, since early XX century,[64] another branch of science and technology developed a broad body of knowledge and applications resulting from the catalytic properties of $CeO_2$.[65] The first industrial application of $CeO_2$ was in 1891, when Carl Auer von Welsbach, student of Robert Bunsen, incorporated it in incandescent mantles for lighting. When combined with other rare-earth metal oxides, cerium glows intensely as soon as it is warmed-up.[66] After this, $CeO_2$ powders in micrometric and submicrometric sizes and more recently in controlled nanoparticulate sizes have been under intense scrutiny as structural and electronic promoters of catalytic reactions. In industry, $CeO_2$ has been most widely used as an active component in processes such as three-way catalysts (TWC) for automobile exhaust-gas treatments, oxidative coupling of methane and water-gas shift reaction (along with other

applications such as polishing agent for optical glasses and silicon wafers, grinding medium for computer parts and camera phone lenses).

Although $CeO_2$ industrial applications are beyond the scope of this review, it is worth to mention, in view to elucidate the mechanisms of $CeO_2$NPs biological activity, the early investigation of $CeO_2$ oxygen storage properties derived from its introduction in TWCs.[67] The purpose of TWC is to promote the simultaneous oxidation of Carbon Monoxide (CO) and hydrocarbons and the reduction of Nitrogen Oxides (NOx). In such a way, the catalyst reduces both the fuel consumption and the emission of soot particles of combustion engines. A summary of the involved reactions is shown in **Figure 1B-D**. In TWC, the catalyst must be an oxygen buffering material, releasing oxygen in a reductive atmosphere and incorporating it by interacting with oxidizing gases present in the mixture, $O_2$ many times. Thus, $CeO_2$, and $CeO_2/ZrO_2$ mixtures, have been widely used as oxygen buffers. Since the 1970s−1980s, the preparation of TWC consisted essentially in the co-impregnation of noble metals, such as Pt, and $CeO_2$ onto the $Al_2O_3$ support.[68] During the mid-1980s, a second generation of $CeO_2$-containing TWCs was developed -with much higher performance- through the improvements in the material preparation to increase the $CeO_2$ content and to optimize the dispersion of the $CeO_2$ particles in the $Al_2O_3$ support.[69]

Therefore, in a context of pursuing antioxidant solutions to many diseases, the rising of studies of the catalytic applications of nanostructured $CeO_2$ and the use of Ln and other NPs for biomedical research, it came the discovery that a cell culture of mixed brain cells incubated with $CeO_2$NPs was still alive and actively signaling after a much longer period than their expected life span. A patent was presented[70] and three abstracts were published.[30] From this, interest in $CeO_2$NPs and how their properties against the accumulation of free

radicals can be applied to medicine, rapidly grew. Since then, many reports and studies are constantly appearing with promising results (**Table 1**).

**Table 1.** Firsts and recent reports using $CeO_2$NPs in different medical areas.

| Year[a)] | Area | Description of the work |
|---|---|---|
| 2003 | First use in nanomedicine | $CeO_2$NPs of less than 20 nm prolonged the lifespan of brain cell cultures, for periods of up to 6-8 months.[30] |
| 2005 | Oncology | Protection from radiation-induced damage: CRL8798 cells (immortalized normal human breast epithelial cell line) and MCF-7 (breast carcinoma cell line) were exposed to radiation. Further treatment with $CeO_2$NPs was shown to confer radioprotection to the normal human breast line but not to the tumoral one.[38a]. Other, more recent, works can be found e.g. in Li et al.[38b] and Nourmohammadi et al.[38c] |
| 2006 | Neurology | $CeO_2$NPs were found to be neuroprotective, limiting the amount of ROS that would decrease viability of nerve cells (HT22 hippocampal nerve cell line).[32a] Neuroprotective effect on adult rat spinal cord neurons demonstrated with electrophysiological recordings of retention of neuronal function in cultured cells isolated from rat spinal cords.[71] Other, more recent, works can be found e.g. in Kalashnikova et al.[32c] and Ranjbar et al.[32d] |
| 2006 | Ophthalmology | $CeO_2$NPs prevented retinal degeneration induced by intracellular peroxides -and thus preserve retinal morphology and prevent loss of retinal function- in an *in vitro* primary cell culture of dissociated cells of the rat retina and an *in vivo* albino rat light-damage model injecting the suspension of $CeO_2$NPs into the vitreous of both eyes.[31a] Other, more recent studies, can be found e.g. in the works of Cai et al.[31b, 31c] |
| 2007 | Cardiology | Intravenously injected $CeO_2$NPs in a transgenic murine model of cardiomyopathy reduced the myocardial oxidative stress, the endoplasmic reticulum stress and suppress the inflammatory process, conferring protection against progression of cardiac dysfunction.[34] |
| 2009 | Chronic inflammation | In vivo study show $CeO_2$NPs potential to reduce ROS production in mice states of inflammation and hence proposed as a novel therapy for chronic inflammation.[15a] |
| 2011 | Diabetes | A combination of $CeO_2$NPs and sodium selenium was beneficial to diabetic rats.[35a] Another, more recent, work can be found e.g. in Khurana et al.[35b] |
| 2013 | Hepatology | CeO2NPs showed similar performance as N-acetyl cystine, a common therapeutic to reduce oxidative stress, in mice with induced liver toxicity (by $CCl_4$).[72] Other, more recent works can be found e.g. in Adebayo et al.[37b] and Fernandez-Varo et al.[37c] |
| 2014 | Regenerative Medicine and tissue engineering | The capacities of $CeO_2$NPs to achieve functional restoration of tissue or cells damaged through disease, aging, or trauma through enhancing long-term cell survival, enabling cell migration and proliferation, and promoting stem cell differentiation were reviewed in the work of Das et al.[39] Another more recent work can be found e.g. in Marino et al.[40] |
| 2017-2019 | NPs in the space | $CeO_2$NPs to counteract the detrimental effects of microgravity-induced oxidative stress.[41] |

[a)]*To the best of our knowledge, we briefly describe here the firsts reports, and more recent ones, that apply the therapeutic potential of $CeO_2$NPs in nanomedicine research. We apologize in advance if other contributions were before the ones here listed as the first one.*

## 3. Liver as a testing field for nanomedicine. The case of $CeO_2$NPs

The application of $CeO_2$NPs in medicine is still a recent research area that needs more work to be done before it can fulfill its full potential. For something that still has to be fully

understood and developed, a good starting point is *what it is really known*. When studying the safety, pharmacokinetics, and biodistribution of nanoparticulate materials in the body, it is already known that the liver and spleen are the major receptor sites after NPs administration (≈90 % of the dose administered), followed by the kidneys (≈9%) and other organs of the reticulum endothelial system, which act as NPs collectors.[73] Indeed, $CeO_2$NPs are not an exception and plenty of studies confirm their passive accumulation in the liver. Hence, the liver represents a major testing field for the study of the pharmacokinetics and the therapeutic effects of $CeO_2$NPs. Additionally, it is well-known the role of ROS in the genesis and progression of liver diseases such as non-alcoholic fatty liver disease (NAFLD) or hepatocellular carcinoma.[74] Therefore, the knowledge acquired here will also pave the way for further application of this and other NPs, and NPs in general, in other organs when properly targeted therapies will be developed.

Thus, in this section, we aim to review different studies of $CeO_2$NPs biodistribution, toxicity and therapeutic effects in different *in vitro* and *in vivo* experimental models of liver disease. The doses and types of $CeO_2$NPs used (size, surface state, their source -either commercial or synthesized in the laboratory-, etc.) are also detailed for each report to better understand the results obtained.

### 3.1. Biodistribution and final fate. Liver as passive target. Kuppfer cells or Hepatocytes?

As said, there is a unanimous agreement that the liver and spleen are the major passive target of $CeO_2$NPs. In an exhaustive biodistribution study, Yokel et al. administered high intravenous (i.v) doses of $CeO_2$NPs (5, 15, 30 nm, 100 mg/kg; 55 nm, 50 mg/kg; all citrate capped) into Sprague Dawley rats and evaluated Ce biodistribution after 1 hour, 20 hours and 30 days.[75] Again, liver and spleen contained a large percentage of the dose and there was no significant decrease of Ce over time. Interestingly, liver contained significantly more of the

total dose of the 5 than 30 nm $CeO_2$NPs at 20 hours, and the spleen contained significantly more of the 15 nm than the 5 nm ceria at 30 days, suggesting preferential accumulation of the smaller (5 nm) NPs by the liver and the larger (15 and 30 nm) by the spleen. In a similar study, these authors evaluated the biodistribution after 1h and 20 h of $CeO_2$NPs (30 nm; 0, 50, 250 or 750 mg/kg) following i.v. administration to Fisher 344 rats.[76] Results showed once more that the liver and spleen were the main targets and no major systemic injury was observed after 20 hours of a single dose of i.v. $CeO_2$NPs infusion. In this work, a faster accumulation rate in the spleen at short times (first hours) and a decrease of Ce in the spleen correlated with an increase of Ce in the liver over time was observed. Intracellular $CeO_2$ agglomerations were observed in both Kupffer cells and hepatocytes. $CeO_2$NPs produced a dose- and time-dependent increase of activated Kupffer cells, evident after 20 h at the 250 mg/kg and 750 mg/kg doses.[76] Results from our groups after i.v. administration of albumin stabilized 4 nm $CeO_2$NPs at 0.1 mg/kg of body weight (bw), twice a week during two weeks in control and fibrotic rats, showed most of the Ce detected in the liver (84% of the total dose of Ce collected).[37a] Furthermore, more than 75% of the initial Ce was still detected 8 weeks after administration.

However, once accumulated in the liver, results in the literature are less coincident regarding the cell types, and subcellular localization, in which $CeO_2$NPs are found. Hirst et al. carried out i.v. administration of $CeO_2$NPs (3-5 nm) to C57BL/6 mice (single dose of 0.1 mg/kg or 0.5 mg/kg) that were sacrificed after a week.[15a] Another mice group with an additional second dose (0.1 mg/kg or 0.5 mg/kg) administered at day 15 were sacrificed at day 30. In both cases, results showed that $CeO_2$NPs were well tolerated. The presence of randomly scattered $CeO_2$NPs within hepatocytes was observed using TEM images. Tseng et al. also evaluated biodistribution employing a high dose of $CeO_2$ nanocubes (85 mg/kg, 30 nm, citrate capped) into Sprague Dawley rats.[77] These $CeO_2$NPs were observed mainly in Kupffer cells

1 hour after infusion, and ultrastructural analysis after 30 and 90 days revealed $CeO_2$ accumulations in Kupffer cells, stellate cells, and hepatocytes. In another study by the same group, a single i.v. injection of a high dose $CeO_2$NPs (5 nm, citrate capped, 85 mg/kg) was given to Sprague Dawley rats and biodistribution was evaluated after 1 hour, 20 hours and 720 hours (30 days).[78] Ce was initially observed in Kupffer cells with subsequent bioretention in parenchymal cells, hepatocytes, and hepatic stellate cells. A study from our groups showed that at subcellular level $CeO_2$NPs were mainly located inside endosome-like bodies in human hepatocellular carcinoma (HepG2) cells.[79] In this work, $CeO_2$NPs were also observed attached to the outer leaflet of the plasmatic membrane and free in the cytoplasm whereas mitochondria, endoplasmic reticulum, and the nucleus appeared normal.

Results from other exposure routes than i.v. also show preferential accumulation in the liver although in less amount due to NPs retention at the portal entry. For instance, Modrzynska et al. evaluated Ce liver deposition after 1, 28 or 180 days of intratracheal instillation of 162 μg of $CeO_2$NPs (79 nm) in C57BL/6 mice.[80] Ce concentration increased over time and the translocation to the liver was 3% of the initial pulmonary dose after 180 days. Almost all the Ce detected beyond the airways was in the liver. Hirst et al. studied $CeO_2$NPs (3-5 nm) administration to CD-1 mice perorally, i.v. or intraperitoneally (i.p.) (weekly for 2 or 5 weeks; 0.5 mg/kg).[72] I.v. administration resulted in the greatest deposition, followed by i.p. and peroral. In both i.v. and i.p. administration, the liver, and the spleen had the highest concentration of $CeO_2$NPs as measured per gram of tissue. Perorally administered mice had very few $CeO_2$NPs deposition. In this study, no liver toxicity was observed regardless of the administration route. In another study, Molina et al., compared the bioavailability, tissue distribution, clearance and excretion of radioactive $^{141}Ce$ after intratracheal instillation, gavage, or i.v. injection of neutron-activated $^{141}CeO_2$NPs and $^{141}CeCl_3$ in Wistar rats.[81] As expected, i.v. administered $CeO_2$NPs were predominantly accumulated in the liver, where

they were retained for at least 28 days. Orally administered $CeO_2$NPs had low absorption from the gastrointestinal tract and rapid elimination through feces. Intratracheal administered $CeO_2$NPs showed minimal extrapulmonary accumulation. Similarly, in the case of inhalation, exposure of Sprague Dawley rats to combustion-generated $CeO_2$NPs (25 and 90 nm bimodal distribution), Ce was predominantly recovered in the lungs and feces, with extrapulmonary organs contributing less than 4 % to the recovery rate.[82] Recently, $CeO_2$NPs uptake by ex-vivo perfused human livers has been demonstrated by our groups.[37c] After administration, most of the $CeO_2$NPs were readily accumulated in the liver and found both free and within intracellular single-membrane endosome-like organelles, while some were observed inside blood vessels, space of Disse and endothelial and blood circulating cells.

Regarding the potential toxicity in the long-term and the final fate of NPs intended for medical applications, results and data are scarce. This could be most likely due to the cost of maintenance of the animal models and the limited possibilities of tracking nanomaterials over long periods of time.[83] Here, the use of $CeO_2$NPs may benefit from the knowledge acquired with other colloidal inorganic NPs studied for longer times. For instance, Sadauskas et al.[84] in a study aiming to at revealing the fate of 40-nm AuNPs after intravenous injections found that the fraction of Kupffer cells containing AuNPs gradually decreased to about one fifth after 6 months and that at the end of the study only fewer macrophages accumulated AuNPs in growing clusters. However, there are fewer reports of the long term effects for the case of $CeO_2$NPs, mainly addressing the effects of commercial $CeO_2$NPs after inhalation exposure.[85] One of the most comprehensive was a 2-year combined chronic toxicity developed at BASF SE (Ludwigshafen, Germany). Carcinogenicity studies were performed according to the Organisation for Economic Co-operation and Development (Test Guideline 453). In the course of this study, the effects of the $CeO_2$NPs (40 nm) dosed at 0.1, 0.3, 1, and 3 mg/m³ upon 3- or 6-month inhalation exposure to rats (5 to 7 weeks old female Wistar rats) was

assessed. Results showed that $CeO_2$NPs did not elicit significant genotoxicity in the alkaline comet assay and micronucleus test.[86] However, $CeO_2$NPs caused inflammatory and oxidative stress reactions in the respiratory tract by the release of inflammatory mediators, pointing out that signs for long-term effects still need to be further evaluated.[87]

The low clearance rate in the liver is shown e.g. for AuNPs and it has been also reported in the case of $CeO_2$NPs (see e.g the mentioned studies of Molina et al.[81] and Modrzynska et al.[80]), while others have reported bioprocessing and/or dissolution and elimination without toxicity.[88] In this last case, routes of excretion of NPs from the body are feces and urine. In this context, it is important to mention that the degradation and dissolution of small NPs in biological environments is well described.[89] In the case of the liver, Muhammad et al. reported slow dissolution and biotransformation of $CeO_2$NPs in physiological media.[89d] It is also noteworthy that in the case of $CeO_2$NPs recent studies suggest these modifications and evolution of $CeO_2$NPs within the liver, indicating in vivo NP dissolution and bioprocessing. Graham et al. reported changes in the $CeO_2$NPs within the liver 90 days after i.v. administration of $CeO_2$ nanocubes (30 nm; 85 mg/kg/) into Sprague Dawley rats.[90] Specifically, after 90 days of residence in the liver, a “second generation” of smaller $CeO_2$NPs was observed, with higher redox activity. This study used a high amount of $CeO_2$NPs, well above the usual therapeutic dose, but suggests that $CeO_2$NPs may undergo *in vivo* processing inside the liver causing a shift toward smaller particle size and increased reactive surface area. In another study by the same authors, using advanced electron microscopy methods, $CeO_2$NPs bioprocessing in the liver and spleen of Sprague Dawley rats receiving i.v. infusion of 85 mg/kg nanoceria (30 nm) was evaluated.[91] In agreement with previous observations, particles were also observed in the liver and spleen up to 90 days post-infusion. Tissue granulomas were observed, mainly in the spleen but also in the liver, which were considered to be the result of the high i.v. exposures and not to be expected at lower

doses. Note that these NPs were relatively large in size and with a considerable aggregation state, both slowing down dissolution. Furthermore, in the mentioned work of Modrzynska et al.[80] the observed NPs in the liver were found to decrease their sizes over time, possibly indicating NP degradation.

### 3.2. $CeO_2$NPs are not toxic in vitro and protect hepatic cells from induced cellular damage

*In vitro* studies evaluating toxic or therapeutic effects have been performed in different cell types, including human cells. Again, doses used together with NPs characteristics are important when considering the biological effects. As in the *in vivo* case (section 3.3.), higher doses of $CeO_2$NPs can compromise cell viability. For instance, Kitchin et al. evaluated the potential hepatotoxicity in human liver HepG2 cells of a 3-day exposure to two commercial $CeO_2$ nanomaterials (8 nm and 58 nm) at 3 μg/ml or 30 μg/ml doses.[92] It was observed an increase of <1.5-fold in 11 of 24 fatty acids when cells were treated with $CeO_2$NPs at 3 μg/ml and around 2 fold increase in 20 of 24 fatty acids when cells were incubated with the same NPs at 30 μg/ml. In contrast, an increase of only one fatty acid (1.4-fold) was observed when cells were incubated with 58 nm $CeO_2$NPs at 30 μg/ml. The same study observed a reduction in some glutathione and gamma-glutamyl metabolites when cells were treated with 8 nm and 58 nm $CeO_2$NPs at 30 μg/ml, although this was not observed when cells were treated with 8 nm $CeO_2$NPs at 3 μg/ml.[92] Similar results were also observed by Kitchin et al. in another study with HepG2 cells exposed up 3 days to five different commercial $CeO_2$ nanomaterials (30-100 μg/ml, sizes ranging from 15 to 213 nm).[93] Metabolomic assessment of exposed cells showed an increased concentration of fatty acids, monoacylglycerols, maltotriose, and reduced S-adenosylmethionine.

Doses higher than 50 µg/ml were found to induce cytotoxicity in other works. For instance, Cheng et al. using concentrations ranging from 0 to 200 µg/mL of hexahedral $CeO_2$NPs (20–30 nm) observed that concentrations higher than 50 µg/mL induced morphological damage, apoptosis and reduced viability in human hepatocellular carcinoma SMMC-7721 cells.[94] In these conditions, $CeO_2$NPs increased the production of ROS and malondialdehyde (MDA), reduced the activity of SOD, GSH peroxidase and catalase and increased the phosphorylation levels of ERK1/2, JNK, and p38 MAPK. Another study showed that different concentrations and forms of $CeO_2$NPs presented different toxicity on HepG2 cells.[95] Specifically, the effects of three types of $CeO_2$NPs with different morphologies (cube 20 -50 nm, octahedron 10-30 nm and rod-like crystals 8nm x 100-400 nm) in HepG2 cells were compared at concentrations ranging from 6.25 to 100 µg/mL. Significant changes in cell morphology were observed from doses of 50 µg/mL and 100 µg/mL.[95] Experimental data obtained in our laboratories confirm these results.

Conversely, under pathological stimuli, antioxidant activity and protective cellular effects of $CeO_2$NPs have been observed *in vitro* on hepatic human cells, usually at doses lower than 100 µg/ml. For instance, at 100 µg/ml of $CeO_2$NPs (4nm), Oro et al. reported inhibition of intracellular ROS formation in HepG2 cells treated with $H_2O_2$.[37a] Also, protective effects of $CeO_2$NPs treatment (8.5 µg/ml) against hyperglycemic induced injury in HepG2 cells incubated in medium with 50 mM of glucose were described.[96] In these conditions, Shokrzadeh et al. showed that $CeO_2$NPs decreased glucose-induced cytotoxicity, ROS production, and lipid peroxidation.[96] In another study, $CeO_2$NPs used at concentrations as low as 1 ug/ml increased viability and decreased oxidative stress of RAW264.7 macrophages exposed to LPS.[97] A more recent work studied the effects of $CeO_2$NPs in human hepatic cells WRL-68, a HeLa derivative cell line, after inhibition of catalase with 3-Amino-1,2,4-Triazole (3-AT). When these cells were incubated with $H_2O_2$, addition of $CeO_2$NPs (1.9 nm,

100 uM (=17 ug/ml) or 150 uM (=25.5 ug/ml)) improved cell viability and decreased cellular ROS.[98] We have observed that $CeO_2$NPs (10µg/ml; 4nm) reduced fatty acid content in human hepatic cells HepG2 cultivated under steatosis conditions.[79] Aiming for mechanisms to explain how $CeO_2$NPs protect cells against oxidative stress, we performed phosphoproteomic analysis in those HepG2 cells. Results showed that $CeO_2$NPs reverted the $H_2O_2$-mediated increase in the phosphorylation of peptides related to cellular proliferation, stress response, and gene transcription regulation, and interfered with $H_2O_2$ effects on mTOR, MAPK/ERK, CK2A1, and PKACA signaling pathways.[99]

### 3.3. $CeO_2$NPs are not toxic in vivo at therapeutic doses

Liver toxicity of $CeO_2$NPs in healthy rodents by different administration routes has been extensively evaluated and found to appear mostly when higher doses (tenths or hundreds of mg of $CeO_2$ per Kg of animal) are used, recalling the Paracelsus toxicology maxim “sola dosis facit venenum” (the dose makes the poison). For example, in the mentioned study of Tseng et al.[78] single i.v. injection of a high dose $CeO_2$NPs (5 nm, citrate capped, 85 mg/kg;) was administered into Sprague Dawley rats. Sustained $CeO_2$ bioretention in the liver was associated with granuloma formations. A significant elevation of serum AST was seen at 1 and 20 h, but not at 30 days after $CeO_2$NPs administration, whereas apoptosis was observed at day 30.[78] These authors also observed adverse hepatic effects after the single i.v. infusion of the same mass concentration of $CeO_2$ nanocubes (30 nm, citrate capped, 85 mg/kg) into Sprague Dawley rats. Small granulomas and an increase in apoptotic cell number were observed between days 30 and 90 after infusion. At these time points, fibrosis and necrosis were not observed and only small changes were found in ALT serum levels.[77]

As discussed in section 4, another source of toxicity is NP aggregation. And NP concentration increase NP aggregation exponentially. Nalabotu et al. found that a single intratracheal

instillation of commercial $CeO_2$NPs (20 nm; 1, 3.5 or 7 mg/kg) to Sprague-Dawley rats was associated with liver toxicity after 28 days.[100] Histopathological alterations observed included hydropic degeneration and enlargement of hepatocytes, dilatation of the sinusoids and nuclear enlargement. There was no evidence of granuloma, portal inflammation, fibrosis, or bile duct abnormalities, except for the presence of some local inflammation in the lobules of some animals. Increased serum ALT levels and reduced albumin levels were observed at 7 mg/kg. The authors, though, describe how the NPs agglomerate into micrometric units when dispersed in the saline vehicle (NaCl 0.9%), which recalls the situation of frustrated phagocytosis and asbestosis as a source of chronic diseases.[101]

From oral exposure routes, toxicity can be also observed at those higher doses. Kumari et al. investigated the toxicity of 28 daily oral doses of 30, 300 and 600 mg/kg bw of 24 nm $CeO_2$NPs and 3 µm $CeO_2$ microparticles in Wistar rats.[102] Increased genotoxicity including DNA damage in peripheral blood leukocytes and liver were observed after exposure to $CeO_2$NPs at 300 and 600 mg/kg bw/day. Significant alterations were observed in ALT and LDH activity in serum and reduced glutathione content (GSH) in the liver at 300 and 600 mg/kg bw/day in a dose-dependent manner. The same authors using $CeO_2$NPs with similar characteristics observed acute oral toxicity and microparticle formation in albino Wistar rats at 100, 500, and 1000 mg/kg bw administered through oral gavage. Results revealed that the highest dose of $CeO_2$NPs (1000 mg/kg bw) induced significant DNA damage in leukocytes and liver cells, micronucleus formation and cytogenetic changes in bone marrow. No significant genotoxicity was observed at 500 and 100 mg/kg bw of $CeO_2$NPs. Biochemical assays showed significant alterations in ALT and LDH activity in serum and GSH content in the liver only in the case of the higher dose of $CeO_2$NPs (1000 mg/kg bw).[103]

Conversely, no toxic effects are usually observed in doses of few tenths of mg or µg of $CeO_2$ per Kg of animal body weight. For instance, in the mentioned work of Hirst et al. where the administrations of $CeO_2$NPs (3-5 nm) to CD-1 mice perorally, i.v. or i.p. (weekly for 2 or 5 weeks; 0.5 mg/kg) was studied, no liver toxicity was observed.[72] Hijaz et al. evaluated folic acid conjugated 10 nm $CeO_2$NPs as a therapeutic agent in ovarian cancer and observed that a twice a week i.p. treatment for 4 weeks at 0.1 mg/kg in nude mice was not associated with histological alterations of the liver nor alterations in the plasma biochemical measurements of liver function.[104] Also, i.p. administration of $CeO_2$NPs to healthy Sprague Dawley rats (100 nm; 0.5 mg/kg for two weeks), Albino Wistar rats (25 nm; 0.01 µg/kg; four doses distributed in 7 days) or BALB/c mice (<10 nm; 200 µg/kg; eight consecutive days) did not result in liver toxicity.[37b, 105] An implantation study, aimed to evaluate the biocompatibility of NPs containing biomaterials and devices, showed that local tissue reactions caused by $CeO_2$NPs after 28 days of the implantation were minimal.[106] In this study, $CeO_2$NPs did not show systemic toxicity or *in vivo* micronucleus induction in bone marrow. Chemical analysis showed that $CeO_2$NPs migrated from the implant sites (250 mg per site) at low levels and were deposited predominantly in the liver.

### 3.4. $CeO_2$NPs at work in the treatment of liver diseases

*In vivo* studies also present different shreds of evidence of protective effects of $CeO_2$NPs in liver disease, usually related to the use of substantially lower doses than those used in toxicity studies **(Table 2 and Figure 3)**. Amin et al. evaluated the ability of $CeO_2$NPs to protect against monocrotaline (MCT)-induced hepatotoxicity in Sprague Dawley rats.[107] MCT is a pyrrolizidine alkaloid plant toxin that causes hepatotoxicity in humans and animals. I.p. administration of $CeO_2$NPs (25 nm; 0.01 ug/kg) resulted in the absence of cellular alterations induced by MCT in rat livers examined by electron microscope imaging. Besides, it was observed a decrease in hepatic total GSH, GSH peroxidase, GSH reductase, and GSH S-

transferase and significant increases in the enzymatic activities of hepatic catalase and SOD. This suggests that $CeO_2$NPs are hepatoprotective agents against MCT-induced hepatotoxicity. Remarkably, $CeO_2$NPs not only had a direct effect on decreasing ROS but also modified the transcriptome of immune cells and recruited them to synthesize more SOD and catalase to relieve the tissue from the deleterious inflammatory states. Such induction of immune cell polarization has been observed elsewhere.[18c, 108]

**Table 2.** Studies showing therapeutic efficacy of $CeO_2$NPs in different in vitro and in vivo models of liver injury or disease.

| Type of study | Model | Liver injury/disease | $CeO_2$NPs (size, dose, and administration route) | Reference |
|---|---|---|---|---|
| *In vitro* | HepG2 cells (human hepatic cells) | Oxidative stress ($H_2O_2$) | 4 nm; 100 µg/ml | [37a] |
| *In vitro* | HepG2 cells (human hepatic cells) | Hyperglycemia | 8.5 µg/ml | [96] |
| *In vitro* | RAW264.7 cells (murine macrophages) | Lipopolysaccharide | 4-5 nm; 5-1000 µg/ml | [97] |
| *In vitro* | WRL-68 (human hepatocytes) | Oxidative stress (3-Amino-1,2,4-Triazole) | 1.9 nm; 5-200 µmol/L | [98] |
| *In vitro* | Primary portal endothelial cells | Cirrhotic rats | 4 nm; 1 µg/ml | [18c] |
| *In vitro* | HepG2 cells (human hepatic cells) | Steatosis | 4nm; 10 µg/ml | [79] |
| *In vivo* | Sprague Dawley rats | Hepatotoxicity (Monocrotaline) | 25 nm; 0.01 µg/kg; i.p. | [107] |
| *In vivo* | BALB/c-mice | Hepatotoxicity ($CCl_4$) | 3-5 nm; 0.5 mg/kg; i.p. | [15a, 72] |
| *In vivo* | Albino Wistar rats | Hepatotoxicity (D-galactosamine and lipopolysaccharide) | 25 nm; 0.01 µg/kg; i.p. | [105a] |
| *In vivo* | Sprague Dawley rats | Peritonitis (polymicrobial) | 10-30 nm; 0.5 mg/kg; i.v. | [109] |
| *In vivo* | Wistar rats | Liver fibrosis ($CCl_4$) | 4-20 nm; 0.1 mg/kg; i.v. | [37a] |
| *In vivo* | Wistar rats | Cirrhosis ($CCl_4$) | 4 nm; 0.1 mg/kg: i.v. | [18c] |
| *In vivo* | Wistar rats | Non alcoholic fatty liver disease (MCD diet) | 4 nm; 0.1 mg/kg; i.v. | [110] |
| *In vivo* | Wistar rats | Liver regeneration (acetaminophen) | 4 nm; 0.1 mg/kg; i.v | [111] |
| *In vivo* | Wistar rats | Liver regeneration (hepatectomy) | 4 nm; 0.1 mg/kg; i.v | [112] |
| *In vivo* | Wistar rats | Non alcoholic fatty liver disease (Neonatal monosodium glutamate) | 1-5 nm; 1 mg/kg; oral | [113] |
| *In vivo* | Sprague Dawley rats | Hepatic ischemia reperfusion injury | 10-30 nm;0.5 mg/kg; i.v. | [109] |
| *In vivo* | Sprague Dawley rats | Sepsis (Lipopolysaccharide) | 4-5 nm; 0.5 mg/kg; i.v | [97] |
| *In vivo* | Sprague Dawley rats | Hepatotoxicity (Doxorubicin) | 100 nm; 0.5 mg/kg; i.p. | [105b] |
| *In vivo* | BALB/c-mice | Hepatocellular carcinoma (DEN) | <10 nm; 100-200 µg/kg; i.p. | [37b] |
| *In vivo* | Wistar rats | Hepatocellular carcinoma (DEN) | 4-20 nm; 0.1 mg/kg; i.v | [37c] |

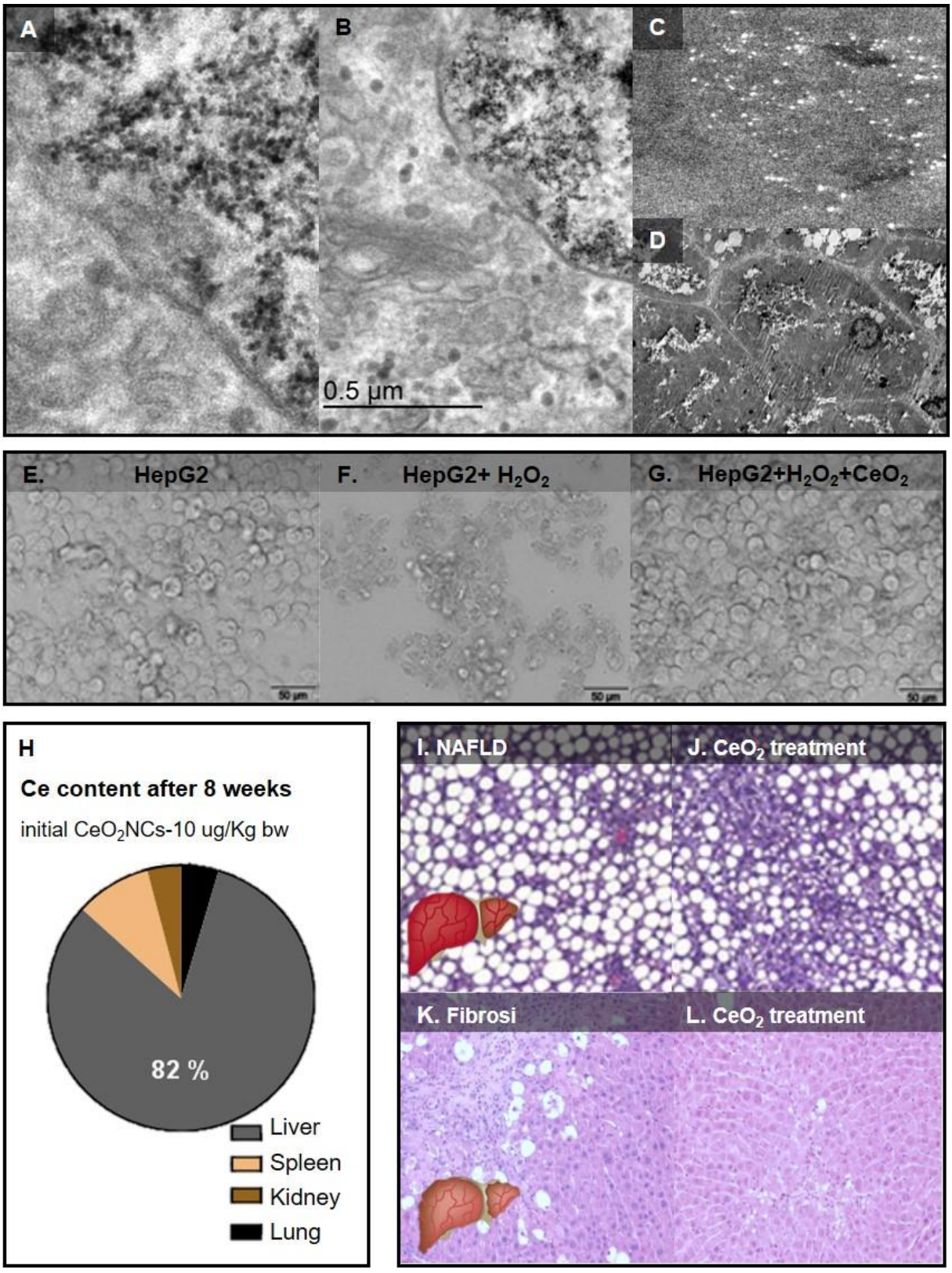


**Figure 3.** Therapeutic effects of $CeO_2$NPs in the Liver. **A-B.** TEM images of $CeO_2$NPs internalized by human hepatic cells (HepG2 cells) revealing the NPs morphology and localization in the cytoplasm. **C.** dark field image of a section of B allowing the NPs to be easily distinguished. **D.** HepG2 cells. E-F. Representative phase-contrast light microscopy images of HepG2 cells after $H_2O_2$ and $H_2O_2$+$CeO_2$NPs treatment showing the protective effects of $CeO_2$NPs under the oxidative stimulus. These results are part of our publication in Carvajal et al.[99] under the terms and conditions of the Creative Commons Attribution (CC BY) license. **H.** Organ distribution upon administration of $CeO_2$NPs after 8 weeks

(n=8). **I-L.** Protective effects in different models in vivo models of rats with NAFLD and Fibrosis. These are preliminary results that led to works in Oro et al.[37a] and Carvajal et al.[110] For the NAFLD case, Wistar rats were subjected to methionine and choline deficient diet (MCDD) for 6 weeks and intravenously treated with $CeO_2$NPs (0.1mg/kg) the weeks three and four of the diet. For the fibrosis case, $CeO_2$NPs (0.1mg/kg) were administered to $CCl_4$-treated rats twice a week for two weeks and $CCl_4$ insult was continued for 8 additional weeks.

In another study, whether $CeO_2$NPs administration (0.5 mg/kg; 3-5 nm) would decrease ROS production in BALB/c-mice treated with $CCl_4$ was evaluated.[72] MDA in plasma was measured as a marker of lipoperoxidation. After 2 weeks of $CCl_4$ administration, MDA was found to be lowered in $CeO_2$NPs treated mice in comparison with non-treated animals. Hashem et al. evaluated the effect of $CeO_2$NPs in hepatotoxicity induced by D-galactosamine and LPS in Albino Wistar rats.[105a] I.p. administration of four doses of $CeO_2$NPs (25 nm; 0.01 μg/kg) decreased the translocation of cytoplasmic Nrf-2 and reduced the levels of iNOS, TBARS (lipid peroxidation marker) and of DNA fragmentation. Also, GSH, GSH peroxidase (GPX1), GSH reductase, SOD and catalase hepatic levels were increased. As well, a significant histological improvement was observed, which suggested antioxidant and hepatoprotective effects in liver toxicity induced by D-galactosamine and LPS. In another study, the administration of a single i.v. dose of $CeO_2$NPs (10-30 nm, 0.5 mg/kg) to Sprague Dawley rats with peritonitis induced by a polymicrobial insult, resulted in improvement of survival associated with modulation of the hepatic inflammatory response and reduced systemic and hepatic oxidative stress.[114] Treated rats presented less sinusoidal dilatation and hepatocyte congestion, reduced hepatic superoxide, lower levels of iNOS expression and protein nitrosylation, less monocyte and lymphocyte extravasation into the peritoneal cavity, decreased infiltration of macrophages into liver, a systemic decrease in the major inflammatory cytokines (IFN-γ, TNF-α, and IL-6) and reduction of GSH S-transferase.

We evaluated systemic and hepatic protective effects of $CeO_2$NPs in Wistar rats after a 16-week $CCl_4$ treatment to induce liver fibrosis.[37a] I.v. administration of $CeO_2$NPs (4-20 nm; 0.1

mg/kg) twice weekly at weeks 8 and 9 reduced portal pressure without affecting mean arterial pressure, decreased serum ALT and AST and reduced steatosis, apoptosis, α-SMA expression and density of infiltrating macrophages/monocytes in the liver tissue. $CeO_2$NPs also reduced hepatic expression of inflammatory mediators such as IL-1β, TNF-α, iNOS and COX-2 and the vasoconstrictor endothelin-1. Interestingly, $CeO_2$NPs significantly reduced hepatic macrophages M1 abundance (pro-inflammatory function; genes TNF-α and iNOS) but did not modify M2 marker expression (macrophages with immunoregulatory function; genes CD163, Arg1 and MRC2). $CeO_2$NPs also reduced hepatic mRNA overexpression of genes related to oxidative stress (Epx), superoxide metabolism (Ncf1 and Ncf2) and ER stress (Atf3 and Hspa5) and rescued messenger expression of PPARγ.[37a] In another study, we evaluated the effect of albumin coated 4 nm $CeO_2$NPs in primary endothelial cells isolated from the portal vein of cirrhotic rats and found that $CeO_2$NPs treatment reduced the proinflammatory state of endothelial cells promoting M2-like phenotype (anti-oxidant/regenerative) and reducing M1 polarization (pro-oxidant/defensive) in macrophages that were exposed to endothelial cell-conditioned medium.[18c] The beneficial effect of $CeO_2$NPs was also linked with differential expression of vasoactive and extracellular matrix remodeling genes that resembled the gene signature found in endothelial cells isolated from healthy animals. Furthermore, cirrhotic rats treated with these $CeO_2$NPs normalized MDA levels in the portal vein and showed and histological improvement of the portal vein endothelium monolayer assessed by scanning electron microscope.

In addition to these, we have also found significant beneficial therapeutic effects of $CeO_2$NPs in experimental models of non-alcoholic fatty liver disease (NAFLD), hepatocellular carcinoma and liver regeneration. $CeO_2$NPs treatment (4 nm, albumin coated, 0.1 mg/kg) of Wistar rats fed with a methionine and choline deficient diet for 6 weeks resulted in reduced liver inflammation and steatosis, suggesting the therapeutic value of these NPs in NAFLD.[110]

In addition, the same type of $CeO_2$NPs administered to Wistar rats with liver hepatocellular carcinoma (induced by a weekly i.p. injection of DEN for 16 weeks) improved overall survival, similar to the multikinase inhibitor sorafenib, which was associated with lower hepatic cell proliferation rate, less macrophage infiltration, specific changes in protein phosphorylation and several lipid components, and reduced levels of the tumor marker α-fetoprotein.[37c] We also assessed the effect of the $CeO_2$NPs treatment on hepatic regeneration in Wistar rats after liver injury by acetaminophen overdose or after 2/3 partial hepatectomy (PHx).[111-112] In both conditions, $CeO_2$NPs treatment stimulated hepatocyte proliferation and decreased early liver damage, indication a beneficial effect of the $CeO_2$NPs in liver tissue regeneration.

To add some more examples, Kobyliak et al. reported anti-inflammatory properties of $CeO_2$NPs on a NAFLD rat model associated with neonatal monosodium glutamate induced obesity.[113b] Oral administration of $CeO_2$NPs (1-5nm; citrate stabilized; 1 mg/kg) for 3 months in 2 two-week courses resulted in a 35% decrease in body weight and a 20% decrease in liver lipids and triglycerides. In another study using this model, the same authors found that orally administered $CeO_2$NPs improved liver histology and decreased lipid peroxidation.[113a] Manne et al. evaluated the protective effects of $CeO_2$NPs administration on hepatic ischemia reperfusion injury in Sprague Dawley rats.[109] Partial warm hepatic ischemia was induced during 1 hour followed by 6-hour reperfusion. Prophylactic treatment with $CeO_2$NPs (10-30 nm, 0.5mg/kg), i.v. administered 1 hour before the hepatic ischemia and reperfusion, decreased serum levels of hepatocellular injury markers (ALT and LDH) and hepatocyte necrosis, preserved normal histological hepatocellular architecture and reduced several serum inflammatory markers (macrophage-derived chemokine, macrophage inflammatory protein-2, KC/GRO, myoglobin and plasminogen activator inhibitor-1). These results suggest that $CeO_2$NPs can be used as a prophylactic agent to prevent hepatic injury associated with graft

failure. I.v. injection of agglomerates of 4-5 nm $CeO_2$NPs (0.5 mg/kg; polyphenol stabilized) in Sprague Dawley rats with LPS-induced sepsis, reduced mortality, liver apoptosis, and hepatic iNOs and HMBG-1, showing potential use of $CeO_2$NPs as a healing agent for liver sepsis.[97]. I.p. administration of $CeO_2$NPs (100 nm; 0.5 mg/kg once a week) to Sprague Dawley in rats simultaneously treated with doxorubicin for two weeks reduced the hepatic toxicity of this chemotherapeutic agent as assessed by histological and structural studies and decreased AST, ALT and MDA levels.[105b] The hepatoprotective potential of $CeO_2$NPs (<10 nm) was also assessed in BALB/c mice with diethylnitrosamine (DEN) induced hepatocellular carcinoma that were pretreated i.p. with $CeO_2$NPs (100 μg/kg or 200 μg/kg) daily for eight consecutive days.[37b] DEN (200 mg/kg) was administered 48 h before the animals were sacrificed. Results show that $CeO_2$NPs attenuated the activities of antioxidant enzymes and expression of Bcl-2 and COX-2 suggesting protection from DEN-induced liver damage via antioxidative activity.

## 4. $CeO_2$NPs in medical applications. Advantages and proposed mechanisms of action

Despite the described beneficial effects of $CeO_2$NPs in many medical conditions, the in vivo mechanisms are not yet totally elucidated and they are difficult to clarify via biological experiments.[20b] Following the discovery of the therapeutic potential of $CeO_2$NPs, it was rapidly thought that they could provide to the field of medicine an effective long-lasting antioxidant compound for the treatment of a broad spectrum of diseases associated with free radical production, especially in diseases related to chronic inflammation and aging.[44, 60] This has been explained by the capacity of $CeO_2$NPs to participate in biological processes mimicking the activity of enzymes such as catalase,[115] SOD[38a, 116] and peroxidase.[117] Afterward, other NPs, mainly $TiO_2$ and $Fe_3O_4$, have been found to be useful in similar applications.[11-12] Further, their participation in different processes addressing the redoxome

was proposed and their intended applications were expanded towards the modification of pathological microenvironments.[18] In this section, their advantages and proposed mechanisms of action are reviewed, focusing on the case of $CeO_2$.

In section 2 it has been introduced the huge rise of popularity of antioxidants since the 1990s and how classic antioxidant substances-such as SOD, ascorbic acid, resveratrol, colchicine, eugenol or vitamin E- have shown limited success in clinical applications, in what has been called the antioxidant paradox.[118] Even more, they have raised controversies after several unsuccessful clinical trials.[61-62] Several shortcomings of those antioxidant agents may account for these failures. One of them is the to-date inability to design efficient antioxidants with targeted and controlled activity. In many clinical trials, the type and dosage of antioxidants did not address the oxidative stress in a tissue- or cell-specific manner (i.e., on target) and therefore did not produce any effect or even contrary effects.[119] Another factor is the limited reaction capabilities of antioxidant molecules, which often scavenge only one single free radical before being inactivated. This is also related to the reaction environment. For instance, while vitamin C acts in the intracellular and extracellular environments, vitamin E acts in the membrane. $CeO_2$, in its NP form, can overcome these drawbacks **(Table 3)**. First, because NPs can be easily functionalized with targeting peptides or molecules and thus designed to have a controlled biodistribution. Although these developments in the case of $CeO_2$NPs are still incipient, some studies already show this possibility, e.g. the works of Li et al.[120] and Xu et al.[121] Second, because $CeO_2$NPs have a long-lasting antioxidant activity due to the high number of reactive sites. This is a major difference between classic antioxidants and $CeO_2$NPs. Whereas the former are quickly oxidized (metabolized), $CeO_2$NPs, may work without being entirely consumed during the reaction. Thus, even at low doses, they can be more effective and with sustained activity over time. Finally, limited by the low $O_2$ concentration inside the body, $CeO_2$NPs only scavenge free radicals when they are in excess,

thus acting as a redox buffer,[10] i.e. $CeO_2$NPs are only "active" in the presence of pathological ROS levels.

**Table 3.** Summary of the advantages of $CeO_2$NPs respect classic antioxidants.

| Classic antioxidants | $CeO_2$NPs |
|---|---|
| No targeted activity. | It can be functionalized, controlled biodistribution. |
| Limited activity: often scavenge one free radical. | Multienzymatic: catalase-like, SOD-like, peroxidase-like activities, NO scavenging, etc. and can participate in the multiplicity of cross-reactions between ROS and inflammation. |
| Limited activity: they are metabolized; after reaction become inactivated. | Not entirely consumed during reaction and thus can work at low doses. |
| Limited activity: short half-life. | Long residence time in tissue. |
| No controlled activity (they become inactivated after reaction). | ROS buffers: only act in conditions of ROS overproduction. |
| Safe | Safe (degraded in innocuous $Ce^{3+}$ ions and expulsed from the body). |

A third reason that accounts for the antioxidant paradox is the limited understanding of the ephemeral nature of ROS/NOS, and also of the interdependence between oxidative stress and inflammation. [119, 122] To add more complexity, the network of antioxidants is complex itself and interrelated (for instance, SOD can catalyze but it, in turn, produces another ROS, $H_2O_2$, as a product).[123] Briefly, ROS and NOS are a variety of molecules including superoxide, $H_2O_2$, hydroxyl free radical, nitric oxide, peroxynitrite, and hypochlorous acid. They are produced naturally as a result of cell metabolism and have an important role in a wide variety of cellular responses, including cell growth, immunity, control of hormone concentration and enzymes activity.[124] The physiological functions of ROS are possible thanks to redox homeostasis, i.e, the presence of a balance between ROS formation and its elimination by endogenous antioxidant systems, mainly composed of glutathione peroxidases, SODs, catalases, thioredoxins, and vitamins C and E.[125] When the redox equilibrium is altered (by an increase in ROS production and/or insufficient response of the natural defense systems), the accumulation of ROS leads to DNA damage (by oxidation of nucleotides and induction of

mutagenesis), protein degradation and lipid peroxidation. These reactions ultimately lead to inflammatory processes.[126] Inflammation itself triggers a higher ROS production as a defense mechanism to generate a less biofriendly environment against pathogens, especially by the innate immune system.[127] This, in the case of some channelopathies, results in epilepsy crisis.[128]

Therefore, the excess of ROS induces inflammation. But the reverse sequence of events is also true: inflammation induces ROS to alter immune cells phenotype and activate them in a sort of positive reciprocal feedback loop.[119, 122] During the inflammatory response, phagocytic cells become activated and produce large amounts of ROS and reactive nitrogen and chlorine species to eliminate commensal organisms.[129] And these reactive species diffuse out of the phagocytic cells, inducing in turn oxidative stress and tissue injury what triggers an immune response and more ROS into a vicious loop. Several mechanisms of ROS-induced activation of inflammatory mediators and DNA modifications have been reported.[130] Thus, selection of antioxidants that do not inhibit both processes -ROS production and inflammatory response- or the use of molecules that block some of the oxidative and/or inflammatory pathways but may trigger the others, could account for unsuccessful antioxidants performance in clinical trials.

In this context, $CeO_2$NPs also display superior activity respect the limited reaction capabilities of classical antioxidants. They can act mimicking the activity of many of the different endogenous antioxidant molecules and they can participate in the multiplicity of the cross-reactions between ROS and inflammation at any level which allows disconnecting these two events **(Figure 4)**. During the last two decades, it has been described for $CeO_2$NPs the SOD activity (conversion of superoxide anion into hydrogen peroxide and finally oxygen),[116, 131] catalase activity (hydrogen peroxide into oxygen and water),[115] and peroxidase activity

(hydrogen peroxide into hydroxyl radicals),[117] as well as of NO scavenging ability,[132] among others. Remarkably, these proposed small size NPs become a rather inert material at healthy physiological conditions, slowly dissolving into innocuous cerium ions that are finally expulsed *via* the urinary tract or the hepatic route.[18c, 37a, 89d]

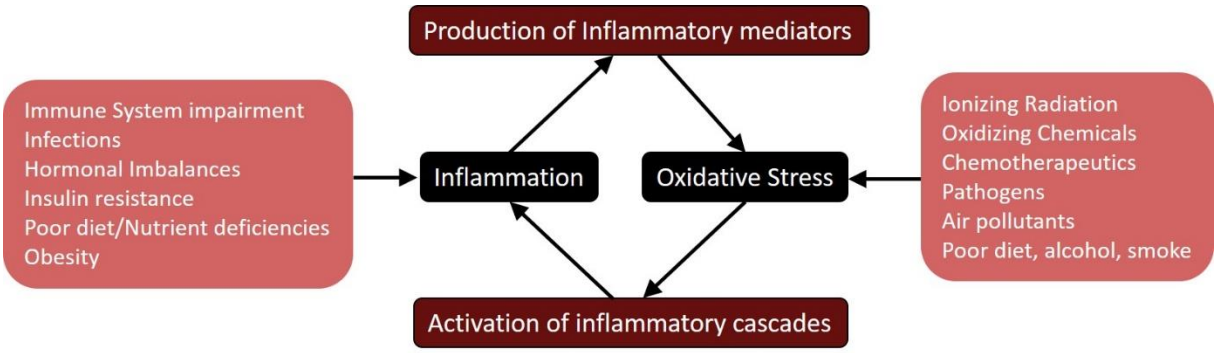


**Figure 4.** Sources of inflammatory and oxidative stress processes and their interrelation. Here, $CeO_2$NPs can act at different levels, breaking the vicious cycle between inflammation and oxidative stress.

This wide free radical scavenging activity is often pictured by an ability of $CeO_2$NPs to participate in those reactions through an auto regenerative redox cycles switching the valence states between $Ce^{3+}$ and $Ce^{4+}$. While most of the research works on the therapeutic activity of $CeO_2$NPs refer to this mechanism, others have been also proposed in biological contexts. Cafun et al. using synchrotron light, observed that the Ce(4f) orbitals remain unchanged even when particle size decreases below 4 nm.[115a] And they remained so, even during the decomposition of $H_2O_2$ (catalase mimetic activity) in model cell culture media. In those high energy resolution experiments, a different mechanism was proposed. Since there is no sign of a redox partner (a local $Ce^{3+}$ site), alterations of the electron density in 5s orbitals suggest that the reaction may take place due to a charge enrichment delocalized over the atoms of the NP, acting as a sort of electron sponge. Thus the NP would be not exactly $CeO_2$ but, $CeO_{1,99}$ (assuming 150 atoms of Ce per NP and one oxygen vacancy), delocalized in such a way that the charge is localized into a Ce atom (responding then as a $Ce^{3+}$ atom) when the $CeO_2$NPs

are analyzed with a scanning tunneling probes.[133] In any case, although the mechanism is still to be completely understood, the use of $CeO_2$NPs already constitutes a disrupting and promising new therapeutic alternative in the many conditions related to chronic inflammation, with activity superior to classic antioxidants.

## 5. Toxicity and Safety. A remaining challenge

Despite the interesting advantages of nanomaterials for medicine and the promising research results obtained, only a few of them have reached the bedside. In the case of inorganic NPs, those are mainly based in iron oxide NPs, e.g. as iron replacement therapy for the treatment of anemia.[29a] Other nanomaterials are already approved e.g. by the American Food and Drug Administration for clinical trials, which mainly include liposomes or organic particles and also some metal and metal oxides NPs such as Au, $SiO_2$ and iron oxide NPs.[29a, 134] Economical and technical aspects slow down the path towards making nanomedicine potentialities a reality. Of course, for any drug development enterprise, investments for new drugs and medical technologies to reach the market and the patients are enormous, derived by the economic conservative exploitation model and the consequent financial needs. Regarding knowledge and technical aspects, a major shortcoming is that the safety of nanomaterials is still a subject of wide debate. In the scientific literature, there are confusing and contradictory results. For instance, for the mentioned case of iron oxide NPs, inhalation exposure of different iron oxide and iron spinel oxide NPs with sizes ranging from 10 to 60 nm have been found to increase levels of DNA strand breaks in an study with female C57BL/6J BomTac mice, showing the potential pulmonary toxicity of these type of nanomaterials.[135]

The case of $CeO_2$NPs is again a paradigmatic example. For this material, along with the works reporting protective effects against ROS overproduction and inflammatory processes,

other studies indicate the opposite, a role of this nanomaterial on promoting oxidative stress, decrease in cell viability through autophagy and apoptosis and inflammation (see e.g. Fisichella et al.)[136] Specifically in liver, as reviewed in section 3, some reports show $CeO_2$ NPs uptake by hepatocytes with beneficial anti-inflammatory effects while others show macrophage (Kupffer cells) uptake with pro-inflammatory effects. In this section, the sources of these discrepancies are discussed and several considerations are proposed.

### 5.1. Different morphological characteristics.

At the source of these discrepancies, there are different factors. One of them is the diversity of materials actually employed. This relates to the multiplicity of works that include a wide variety of different particles in terms of sizes, surfaces states, concentrations, stabilities and so on. In such cases, the results from one study can not be generalized and/or translated to other studies without a careful look at the characterization details of the material. In the case of $CeO_2$NPs, different formulations are presented under the same *$CeO_2$ nanoparticles* or *nanoceria* or *nanocrystalline cerium dioxide* or similar labels **(Figure 5)**. Indeed, the effects of $CeO_2$NPs as "active ingredient" depend on the formulation of the final product in which they are presented. Different reagents and procedures are employed to prepare $CeO_2$NPs yielding NPs with different surface states and/or embedded in colloidal solutions with different stabilizers. For instance, Dowding et al. prepared different samples of $CeO_2$NPs using identical precursor (Cerium Nitrate Hexahydrate) through similar wet chemical process but using different reagents for their synthesis and stabilization: $H_2O_2$, $NH_4OH$, or hexamethylenetetramine (HMT).[137] Results showed that, unlike the other $CeO_2$NPs preparations, HMT- $CeO_2$NPs were readily taken into endothelial cells and reduced cell viability at a 10-fold lower concentration than the others, attributed to HMT. Another example is the preparation of NPs employing intrinsically toxic compounds.

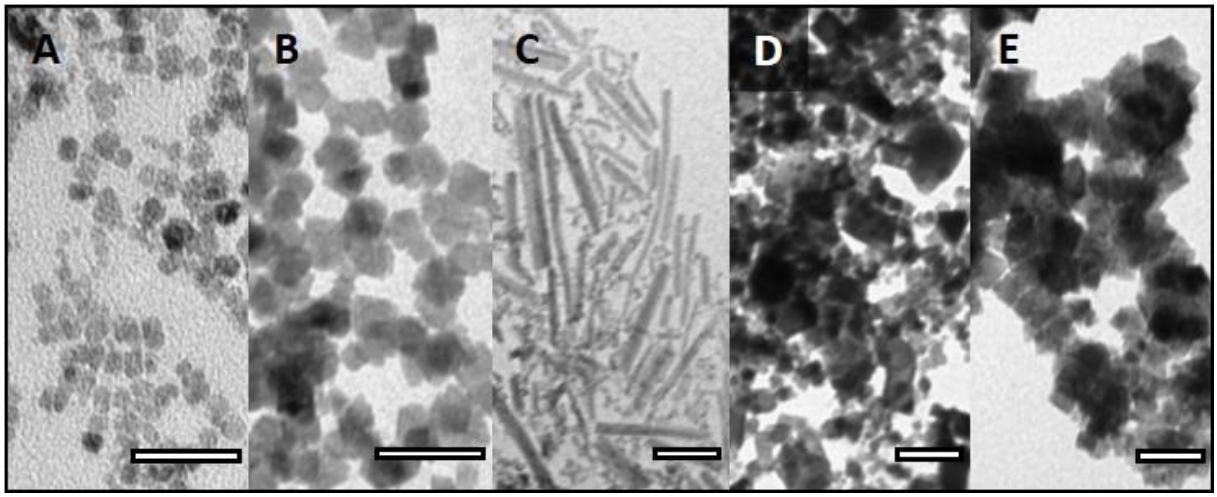


**Figure 5.** TEM images of different samples of $CeO_2$NPs with different sizes, shapes and size distributions, all labeled as $CeO_2$NPs. Scale bars are 20 nm; **A)** 4 nm $CeO_2$NPs, synthesized with $Ce(NO_3)_3$ and TMAOH; **B)** 15 nm $CeO_2$NPs synthesized with $Ce(NO_3)_3$ and HMT; **C)** $CeO_2$ nanorods; **D)** Commercial $CeO_2$NPs in dry form after resuspension in $H_2O$; **E)** Commercial $CeO_2$NPs in dry form after resuspension in Cell Culture Media (CCM).

Different works have attempted to describe trends between NPs morphology, surface characteristics and biological outcome. Asati et al., exposing polymer-coated $CeO_2$NPs with positive, neutral and negative surface charges to normal and cancer cell lines showed the differences in internalization and toxicity.[138] Positive and neutral charged NPs were uptaken by all cell lines studied, while negatively charged NPs were uptaken only in the cases of cancer cell lines. Differences in subcellular localization depending on the NPs surface charge were also shown, being significantly toxic only when they localize in the lysosomes of the cancer cells. Fisichella et al. also showed how surface modifications affected cytotoxicity results.[136a] In this study, non-coated $CeO_2$NPs down-regulated key genes involved in metabolic activity while ammonium citrate capped $CeO_2$NPs did not display any adverse effect at the same concentration. Regarding morphologies, Ji et al. observed that $CeO_2$ nanorods (with different lengths from hundreds of nanometers to micrometers) induced a progressive increase in IL-1β production by generating lysosomal damage while $CeO_2$ nanospheres and shorter nanorods did not show significant toxicity.[139] Here, it is important to note that fiber-like materials deceive the macrophages of the innate immune system during

phagocytosis, leading to chronic inflammation. However, this is the case of microstructures (single crystal or aggregates) rather than isolated nanostructures.[140]

Consequently, the variability of the nanomaterials used and their, sometimes, poorly described characterization are barriers to the development of this multidisciplinary area. This is of high importance in the case of nanometric size NPs, where minor variation in the NPs morphology may have a large impact on the biological outcome. Indeed, to test all the possible variations to have a complete picture of NPs safety aspects is a cumbersome task but different strategies to decrease the burden of work for nanomaterials safety assessment have been proposed and reviewed elsewhere.[27, 141]

### 5.2. Different evolution in biological environments. Extrinsic properties of nanomaterials

Another source of the discrepancies between beneficial and detrimental NPs effects is the different evolution of the actual materials being tested. Cellular environments and physiological media contain different and higher ionic and molecular compositions than the NPs synthesis media. Similarly, there are different redox states (from rather reducing to oxidizing) and different pHs (the late endosome and lysosomes can go down to 5) inside tissues and cellular structures, as well as the presence of nucleophilic species and ionic scavengers. The processes that NPs undergo in these conditions are diverse and a variety of parameters are involved. These have been also described and reviewed elsewhere.[1b, 73a, 142] Generally, it has been described the agglomeration into submicrometric or even micrometric particles,[142b] the corrosion and dissolution into molecular or ionic species,[89a-c, 143] and the surface modifications, particularly the adsorption of proteins or other macromolecules

forming the so-called Protein Corona.[144] In addition, all these processes may take place simultaneously and with different temporal evolutions, which difficult their study **(Figure 6)**.

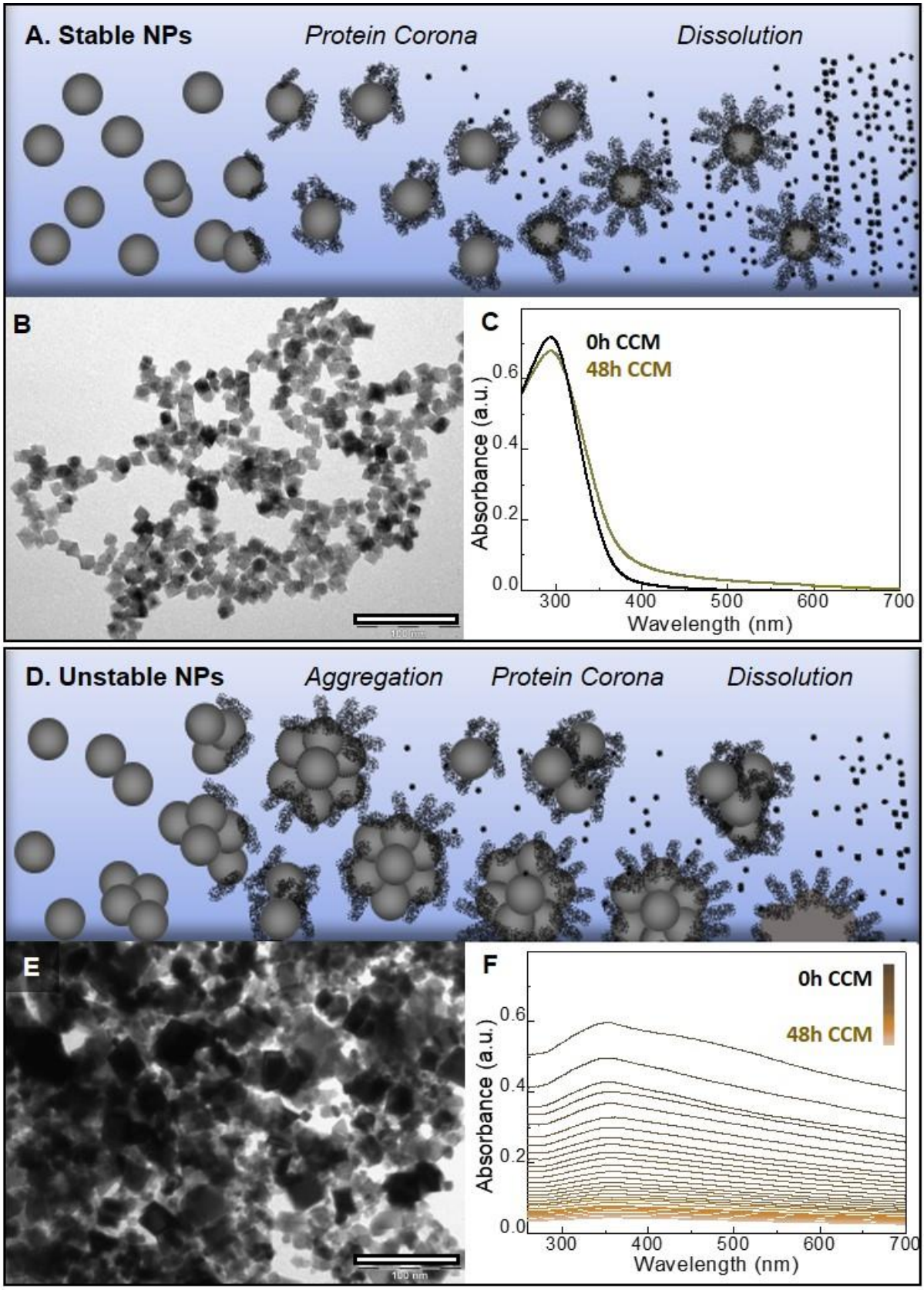


**Figure 6. $CeO_2$NPs from different sources. A.** Schematic representation of the time evolution of

stable NPs dispersed in physiological environment. Protein Corona and Dissolution take place simultaneously and with different time evolutions. Eventually, NPs are dissolved and expulsed by urine as reported for the case of $CeO_2$NPs; **B.** TEM image of 10 nm $CeO_2$NPs synthesized in the laboratory as described in Cafun et al.[115a] Scale bar is 100nm; **C.** UV-VIS spectra of NPs from image B as-synthesized and after 48 hours dispersed in Cell Culture Media (CCM) consisting of DMEM + 10%FBS); **D.** Schematic representation of the time evolution of unstable NPs dispersed in physiological environment. Aggregation takes place at short times, which slows down dissolution, while Protein Corona stabilizes the agglomerates formed initially; **E.** TEM image of commercial $CeO_2$ nanopowders dispersed in CCM and with a nominal size of 25 nm according to the manufacturer. Scale bar is 100nm; **F.** 48 hours evolution of the UV-VIS spectra of NPs from image E dispersed in CCM.

Importantly, all these modifications depend to a large extent on the characteristics of the biological media in which NPs are dispersed. Therefore, their biological effects will depend not only on the NPs intrinsic properties (characteristics such as size and shape) but also extrinsic (characteristics of the exposure media, such as the ionic strength, pH, molecular content, etc). These extrinsic features modify the morphology, surface state and hence, affect the activity, biodistribution, and fate of the NPs, as we reviewed recently.[145] This is especially critical in the case of NPs since their activity depends largely on their surface chemistry and characteristics. Thus, the safe and effective use of promising therapeutic NPs needs not only a proper evaluation of possible unwanted (toxic) effects but also the understanding of their precise evolution and biodistribution (ADME profiles) inside the human body.[142c, 146] In this scenario, the development of reproducible and reliable analytical methods for the dynamic characterization of the evolution of nanomaterials in biological environments is recognized as a pressing need to perform reliable nanosafety studies. This was pointed out e.g. back in 2012 in an editorial of Nature Nanotechnology,[147] and more recently, for the specific case of a type of NPs (nanozymes) by the news and opinion of Ghorbani et al.[24]

The challenges that this characterization involves are exemplified in the recent work of Carlander et al.[148] These authors attempted to test the appropriateness of a physiologically based pharmacokinetic model of $CeO_2$NPs systemic distribution in rats. They used

experimental biokinetic data from the literature and results from research works with $CeO_2$NPs with different sizes, coatings, and doses, administered to rats through various exposure routes. The authors could fit the results into the model in only one specific case, 5 nm $CeO_2$NPs citrate capped. Conversely, the model failed for other types of NPs since, as the authors acknowledge, "overall, the modeling results suggest that the biokinetics of $CeO_2$NPs depend not only on the properties of NPs (size and coating) but also, and even more so, on the exposure conditions (route and dose)".[148]

In this context, it is worth noting that the majority of negative immune effects reported in the scientific literature are related to NPs aggregation and contamination, which cause biological effects independent of the composition, size, and shape of individual NPs.[145-146] For instance, aggregates of $TiO_2$,[149] $Al_2O_3$,[150] and $Fe_2O_3$[151] NPs showed similar toxicity to $CeO_2$NPs aggregates.[100, 152] In the case of $Fe_3O_4$NPs, one of the first inorganic nanomaterial employed in biomedical research,[153] in the same year one report showed promising nerve cell regeneration activity[154] while others found toxicity to neuronal cells.[155] Another example is the mentioned work of Hadrup et al.[135] where the conversion of mass-dose into specific surface-area-dose showed that inflammation correlated with the deposited surface area, highlighting once more that the evolution in the physiological environment is of paramount importance. Furthermore, $CeO_2$NPs have been reported to be pro-oxidant (instead of anti-oxidant) depending on its aggregation state,[137, 152, 156] and chronic exposure to $CeO_2$NPs aggregates was found to be associated with increased levels of ROS and heat shock stress response.[152] In turn, generally, isolated, non-contaminated NPs consistently show no toxicity and, in the case of NPs, displaying therapeutic benefits, e.g. references in section 3.4 and references in the reviews of Wang et al.,[23] Liu et al.,[22] and Ghorbani et al.[24]

Even more, due to their industrial applications, most of the research on the toxicity of $CeO_2$NPs has been done to assess occupational and environmental exposure. In such studies often industrial $CeO_2$ nanomaterials were employed, which were normally polydisperse, poly-aggregated and contaminated. As well, this type of material is often supplied in dry aggregated form, that further aggregate in biological fluids **(Figure 6E-F)**.[142b, 157] In those studies, toxic effects were often found. Nowadays, these toxic effects have been attributed to aggregation and contamination of samples.[146] Besides, in these types of studies, the administered doses are usually higher than those proposed in nanomedicine.

### 5.3. Towards the next generation of NPs for medical applications

The present discussion for the case of $CeO_2$NPs can be extended to other NPs intended for medical applications. For any NP, bioactivity operates on a scale where NP morphology, environment and behavior are strongly coupled. Thus, sometimes results observed have been wrongly attributed to the object tested without a characterization of its evolution *in operando*. Other times, poorly described nanomaterials have been used, which difficult to infer any structure-activity correlation. Nowadays, a large amount of data regarding NPs activity has been gathered but little progress made towards matching expectations since some key parameters such as their stability in the physiological media (agglomeration or degradation) and protein corona formation, the impact of pH and temperature and biodistribution, clearance and excretion routes have not been properly addressed in most of the papers.[24, 146]

In this scenario, providing highly soluble NPs in the physiological media is a requirement for their meaningful and controlled use. As said, this is especially significant in the case of NPs. From one side, and as for any other nanomaterial, because their stability will determine the proper interaction with biological entities. From another side, because processes of

agglomeration, protein corona formation, and dissolution modify surface properties and available surface areas. Advances in NPs preparation are significantly boosting the progress in nanomedicine research and can overcome some of these challenges. For instance, the design of nanostructures with a core-shell architecture is known to improve the physical and chemical properties of the composite by providing a protective interface to the NPs and combining other functionalities on a nanoscopic length scale.[158] Polymeric NPs and liposomes have traditionally been among the most commonly used materials for such purposes (see e.g. Sailor et al).[159] More recently, metal-organic frameworks, dendrimers, and silica-based inorganic hybrid NPs have been explored.[160] However, this surface engineering may lead to the reduction of the surface area available for NP reactivity. One simple and effective solution could be to promote NPs solubility by pre-albuminizing them during the preparation process.[18c, 79] Since the formation of agglomerates in physiological media may occur rapidly, the design of NPs stabilized with albumin prior aggregation during their synthesis allowed to obtain NPs more stable and with higher activity, which recalls the successful case Abraxane®, one of the first approved nanomedicines.[161]

In summary, some critical determinants that need to be carefully addressed to drive the NPs clinical benefits towards their clinical translation are depicted in **Figure 7** and can be grouped into the following: i) the development of ADME and nanopharmacokinetics models for NPs to the understand their precise biodistribution and evolution inside the human body; ii) the application of standardized operating procedures for their dynamic characterization in the physiological media; iii) the consideration of underappreciated parameters, such as the morphological characteristics of different materials labeled uniformly as “nanoparticles” and possible contamination of the samples; iv) the development of well-dispersed NPs in solution and their use at appropriate doses.

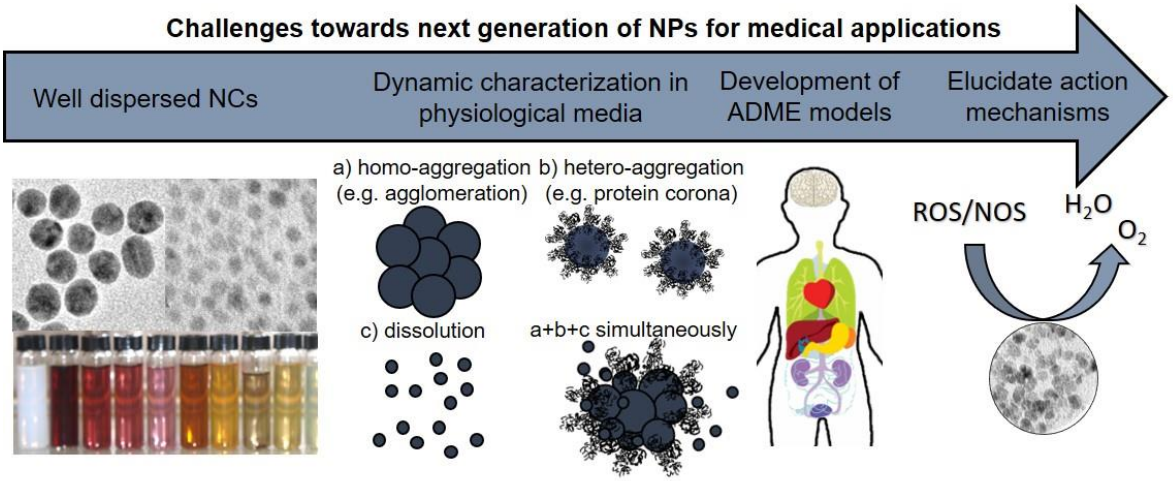


**Figure 7.** Considerations discussed in this work towards the next generation of NPs for medical applications.

**Conclusion and outlook.**

In this review, we have highlighted some general trends after these almost two decades of work with $CeO_2$NPs for medicine, which can be applied to other NPs. First, as other NPs, after i.v. administration they passively accumulate in liver and spleen (up to 90-95% of the administered dose) with a minor fraction identified in the lung and kidneys, and minimal or undetectable in other organs. Thus, it can be easily understood that the liver represents a major testing field for the study of the evolution and therapeutic effects of NPs and their clinical translation. Remarkably, the knowledge gained in the liver would be also of importance for future applications in other organs when properly targeted therapies will be developed.

Following their liver accumulation, it could be concluded that $CeO_2$NPs generally do not show toxicity *in vitro* neither in healthy rodents under standard therapeutic doses and remain in the liver for a long time after their administration (at least months). After this time, $CeO_2$NPs degrades into innocuous $Ce^{3+}$ ions that are expulsed via the kidney. This situation has been also shown for the other proposed NPs.[162] It can also be observed that $CeO_2$NPs only display toxicity in rodents when used at high doses (> tenths of mg of $CeO_2$ per Kg of

animal), while they display hepatoprotection against different induced damages at doses up to 1 mg/kg bw. A similar trend is observed *in vitro*, where NPs used at higher doses, and/or when they precipitate in the cell culture media, compromise cellular viability. Conversely, $CeO_2$NPs usually show cellular protective effects against different insults, at doses from 1 to 100 µg/ml. Thus, NPs biokinetics will depend largely on NPs characteristics, evolution in the physiological media, the dose employed and the exposure route. For all this, we aimed to provide in section 3 the most of the published studies available regarding the characteristics and doses of the NPs used mainly for liver disease treatment, since this information is critical to understand the biological results obtained by independent groups of researchers.

Still, some work needs to be done before the appealing properties of NPs can arrive to patients and society. This has been discussed in sections 4 and 5. First, mechanisms of action are still to be completely elucidated and understood. Most of the works in scientific literature where the antioxidant activity of NPs is intended for medical applications are mainly focused on the therapeutic outcome and much less to the clarification of the mechanisms. Theoretical and experimental work on the performance of NPs in the physiological environment must be coupled with the nanomaterial evolution in the working media and the precise knowledge of their cellular and subcellular distribution. For instance, in the specific case of liver, the different relative hepatic uptake of NPs by hepatocytes and Kupffer cells (which depends on NPs size and solubility, i.e., their colloidal stability) makes it difficult to certainly determine where and how the NPs may function. In addition, a comprehensive evaluation of the safety aspects is needed. Along with toxicity aspects, it must include the NPs in vivo stability and their pharmacokinetics, biodistribution, and fate. Here, one of the advantages of working with inorganic NPs is that they have physical (and chemical) signatures very different from biological tissue so it allows the accurate monitoring of their evolution and biodistribution.

Indeed, biodistribution and fate must be considered in healthy and disease models. A good compilation of available characterization techniques for these purposes has been recently reviewed by Modena et al.[3] Another question that will need further work is whether those described beneficial effects would be good enough and powerful enough to redress biological states also in the long term, or on the contrary, they could pose potential toxic effects.

The next unavoidable step towards the clinical use of $CeO_2$NPs is to have them produced under GMP conditions. Their preparation and development under these conditions are not especially challenging since it can be a simple one-step reaction. The most critical part would be the procurement of GMP reagents for the synthesis since these types of materials are not on the drug discovery pipeline of chemicals producers. The development of a pharmacological product (with the characteristics of isotonic, endotoxin-free, sterile and stable) will also require the strict definition of the NPs characteristics. This refers to their size, monodispersity, NPs purity and colloidal stability, and the presence of excipients and potential by-products. In addition, it would be needed studies of stability (shelf-life) of the product and the tolerance to specifications, aspects that the scientific community seems, at this stage, to be able to address successfully. Here, we would like to note that a combination of simple spectroscopy analysis of the NPs such as UV-VIS, Dynamic Light Scattering, and Z-potential measurements may provide precise signatures of the samples since these techniques are extremely sensitive to NPs alterations. Finally, approval by the regulatory agencies has to be obtained. For this, $CeO_2$NPs will have to follow a similar process as the mentioned case of $Fe_3O_4$NPs, already approved by the EMA and FDA for different medical uses, as contrast agent for MRI (Resovist®), as iron supply in the case of ferropenic anemia (Feromuxytol®), or as hyperthermia agent to treat neuroblastoma (Nanotherm®).

For this translation, and following e.g. FDA guidance for industry on drug products, including biological products, that contain nanomaterials,[163] it is acknowledged that nanotechnology can be used in a broad array of FDA-regulated products, being the active ingredients, carriers or adjuvants and that their inclusion may modify significantly the substance behavior. It is important to note that the FDA does not categorically judge all products containing nanomaterials as intrinsically benign or harmful. It is recognized that the nanoform of a substance may change dissolution rates and that NPs can be passively or actively targeted to different sites within the body. Hence, particular physicochemical analysis is needed to define and control the product and ADME considerations have to be revisited for NPs. Finally, the clinical development of drug products containing nanomaterials should follow all policies and guidelines relevant to clinical safety and efficacy studies as any other substance, taking into account their particular physicochemical properties. All this should be an integral part of the future work where chemists, material scientists, molecular biologists, and medical doctors may work together to make even greater medical achievements.

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**Figure 1.**

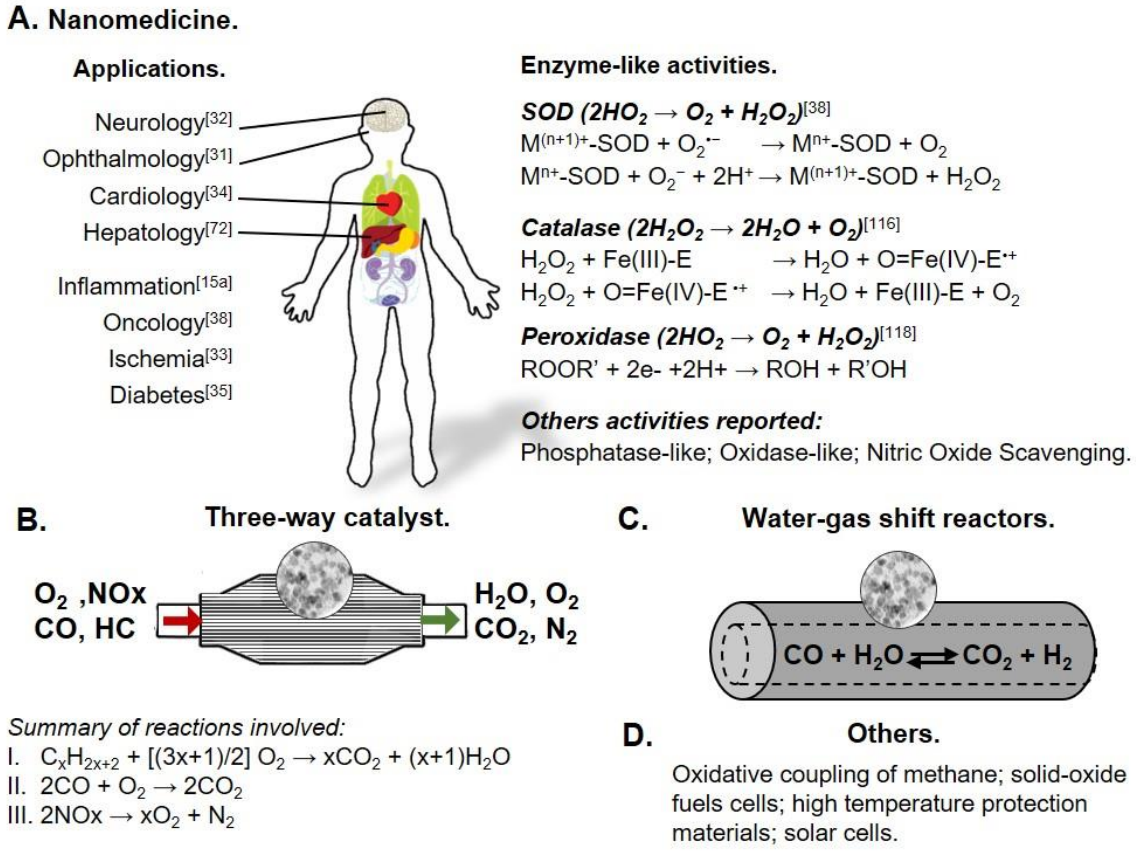


***Figure 1. Different reactions and applications in which $CeO_2$NPs are being proposed or used as antioxidant NPs. A)*** *In nanomedicine research. References are, to the best of our knowledge, the first study reporting the application. We apologize in advance if other contributions were before the ones here listed. Abbreviations: SOD: M=Cu (n=1); Mn (n=2); Fe(n=2); and Ni (n=2); Catalase: Fe(III)-E (heme group iron center attached to catalase; Fe(IV)-$E^{\bullet+}$ (mesomeric form of Fe(V)-E, i.e.,iron not completely oxidized to +V); Peroxidase: The electron donor is very dependent on the structure of the peroxidase. They also may contain in their active site, among others, a heme cofactor or redox-active cysteine or selenocysteine residues.* ***B)*** *In three ways catalytic converters, where I. Oxidation of unburnt hydrocarbons; II. Oxidation of Carbon Monoxide (CO); III. Reduction of Nitrogen Oxides to Nitrogen;* ***C)*** *In water shift reactions and* ***D)*** *Other applications where $CeO_2$NPs are used.*

**Figure 2.**

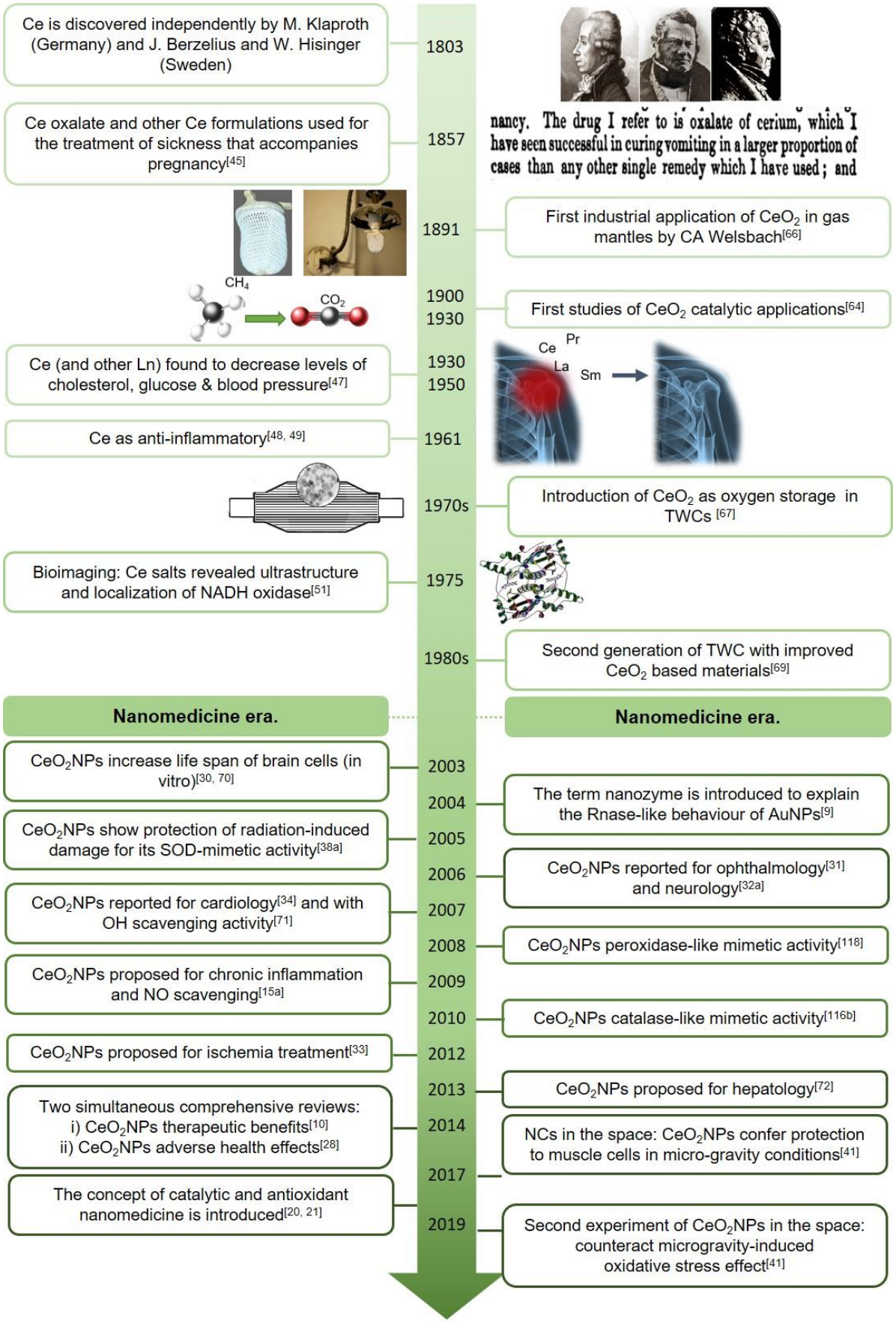


**Figure 2.** Timeline of different achievements using Ce based materials since Ce discovery in 1803.

**Figure 3.**

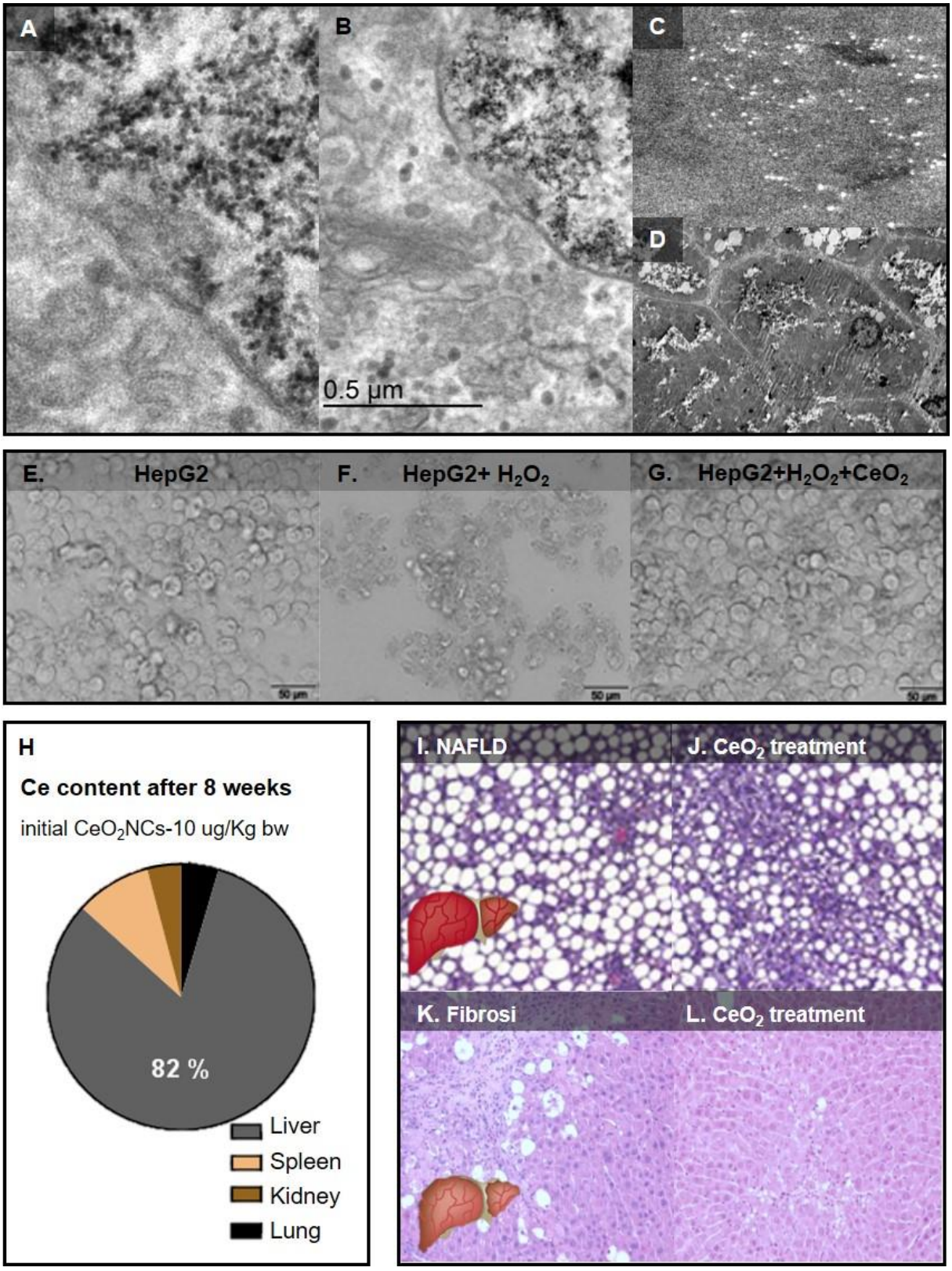


**Figure 3.** Therapeutic effects of $CeO_2$NPs in the Liver. **A-B.** TEM images of $CeO_2$NPs internalized by human hepatocytes (HepG2 cells) revealing the NPs morphology and localization in the cytoplasm. **C.** dark field image of a section of B allowing the NPs to be easily distinguished. **D.** HepG2 cells. E-F. Representative phase-contrast light microscopy images of HepG2 cells after $H_2O_2$ and $H_2O_2$+$CeO_2$NPs

treatment showing the protective effects of $CeO_2$NPs under the oxidative stimulus. These results are part of our publication in Carvajal et al.[99] under the terms and conditions of the Creative Commons Attribution (CC BY) license. **H.** Organ distribution upon administration of $CeO_2$NPs after 8 weeks (n=8). **I-L.** Protective effects in different models in vivo models of rats with NAFLD and Fibrosis. These are preliminary results that led to works in Oro et al.[37a] and Carvajal et al.[110] For the NAFLD case, Wistar rats were subjected to methionine and choline deficient diet (MCDD) for 6 weeks and intravenously treated with $CeO_2$NPs (0.1mg/kg) the weeks three and four of the diet. For the fibrosis case, $CeO_2$NPs (0.1mg/kg) was administered to $CCl_4$-treated rats twice a week for two weeks and $CCl_4$ insult was continued for 8 additional weeks.

**Figure 4.**

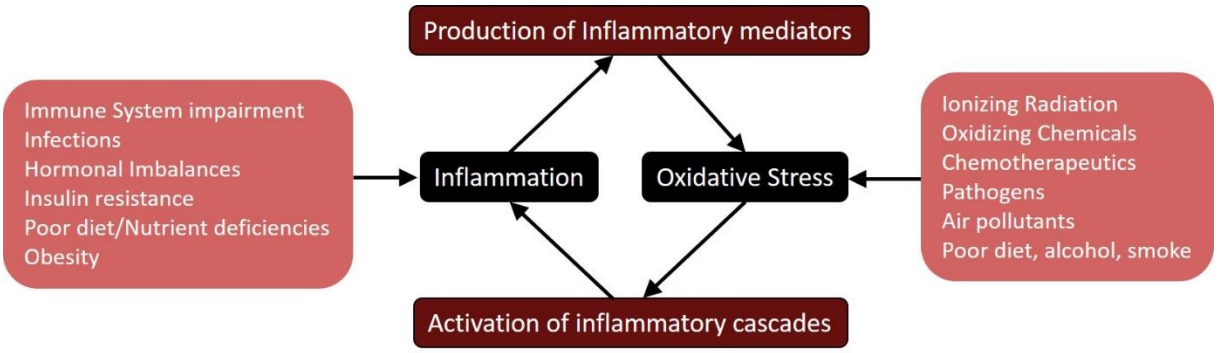


**Figure 4.** Sources of inflammatory and oxidative stress processes and their interrelation. Here, $CeO_2$NPs can act at different levels, breaking the vicious cycle between inflammation and oxidative stress.

**Figure 5.**

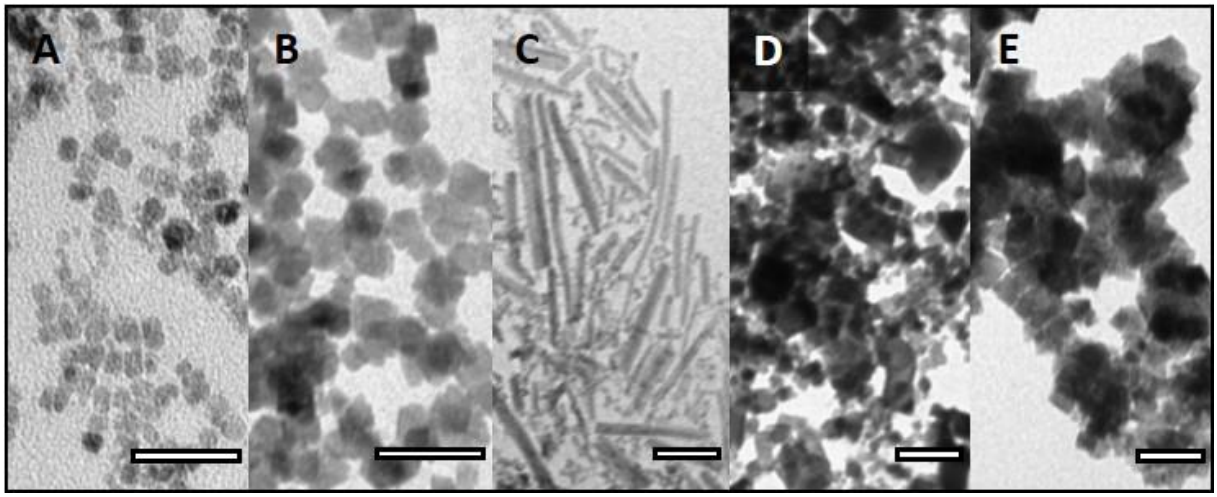


**Figure 5.** TEM images of different samples of $CeO_2$NPs with different sizes, shapes and size distributions, all labeled as $CeO_2$NPs. Scale bars are 20 nm; **A)** 4 nm $CeO_2$NPs, synthesized with $Ce(NO_3)_3$ and TMAOH; **B)** 15 nm $CeO_2$NPs synthesized with $Ce(NO_3)_3$ and HMT; **C)** $CeO_2$ nanorods; **D)** Commercial $CeO_2$NPs in dry form after resuspension in $H_2O$; **E)** Commercial $CeO_2$NPs in dry form after resuspension in CCM.

**Figure 6.**

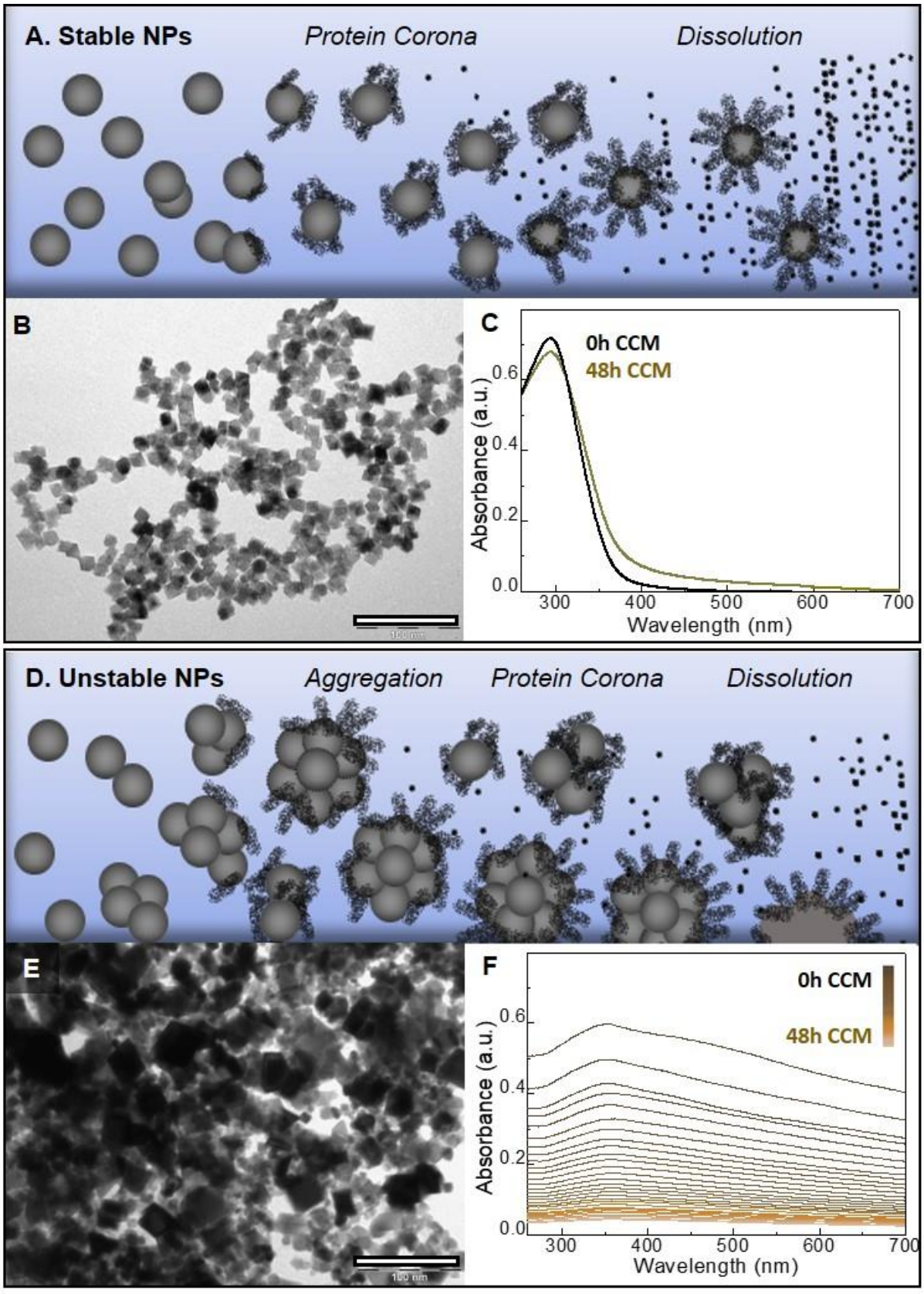


**Figure 6. $CeO_2$NPs from different sources. A.** Schematic representation of the time evolution of stable NPs dispersed in physiological environment. Protein Corona and Dissolution take place simultaneously and with different time evolutions. Eventually, NPs are dissolved and expulsed by

urine as reported for the case of $CeO_2$NPs; **B.** TEM image of 10 nm $CeO_2$NPs synthesized in the laboratory as described in Cafun et al.[129] Scale bar is 100nm; **C.** UV-VIS spectra of NPs from image B as-synthesized and after 48 hours dispersed in Cell Culture Media (CCM) consisting on DMEM + 10%FBS); **D.** Schematic representation of the time evolution of unstable NPs dispersed in physiological environment. Aggregation takes place at short times, which slows down dissolution, while Protein Corona stabilizes the agglomerates formed initially; **E.** TEM image of commercial $CeO_2$ nanopowders dispersed in CCM and with a nominal size of 25 nm according to the manufacturer. Scale bar is 100nm; **F.** 48 hours evolution of the UV-VIS spectra of NPs from image E dispersed in CCM.

**Figure 7.**

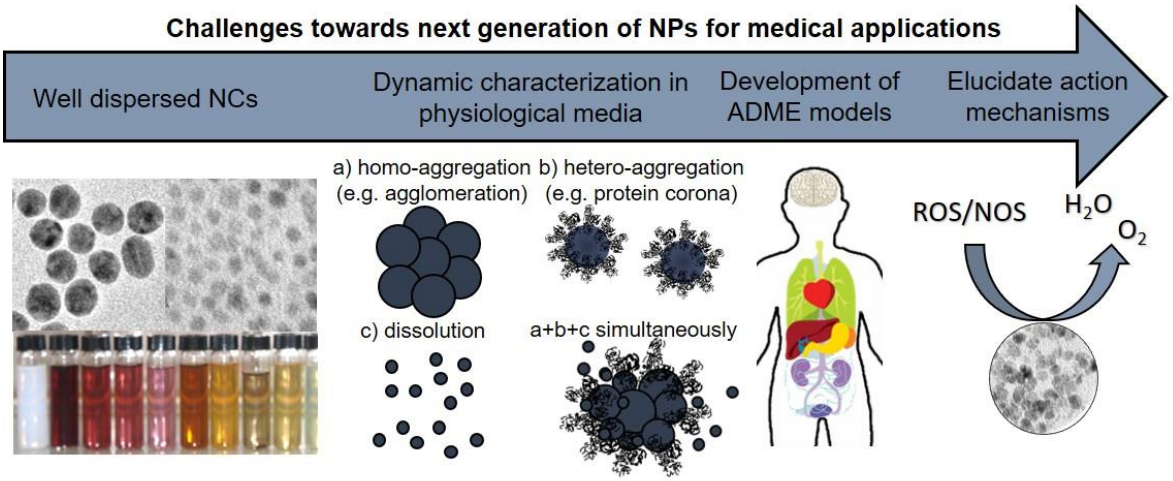


**Figure 7.** Considerations discussed in this work towards the next generation of NPs for medical applications.

## Table 1.

**Table 1.** Firsts and recent reports using $CeO_2$NPs in different medical areas.

| Year[a)] | Area | Description of the work |
|---|---|---|
| 2003 | First use in nanomedicine | $CeO_2$NPs of less than 20 nm prolonged the lifespan of brain cell cultures, for periods of up to 6-8 months.[30] |
| 2005 | Oncology | Protection from radiation-induced damage: CRL8798 cells (immortalized normal human breast epithelial cell line) and MCF-7 (breast carcinoma cell line) were exposed to radiation. Further treatment with $CeO_2$NPs was shown to confer radioprotection to the normal human breast line but not to the tumoral one.[38a]. Other, more recent, works can be found e.g. in Li et al.[38b] and Nourmohammadi et al.[38c] |
| 2006 | Neurology | $CeO_2$NPs were found to be neuroprotective, limiting the amount of ROS that would decrease viability of nerve cells (HT22 hippocampal nerve cell line).[32a] Neuroprotective effect on adult rat spinal cord neurons demonstrated with electrophysiological recordings of retention of neuronal function in cultured cells isolated from rat spinal cords.[71] Other, more recent, works can be found e.g. in Kalashnikova et al.[32c] and Ranjbar et al.[32d] |
| 2006 | Ophthalmology | $CeO_2$NPs prevented retinal degeneration induced by intracellular peroxides -and thus preserve retinal morphology and prevent loss of retinal function- in an *in vitro* primary cell culture of dissociated cells of the rat retina and an *in vivo* albino rat light-damage model injecting the suspension of $CeO_2$NPs into the vitreous of both eyes.[31a] Other, more recent studies, can be found e.g. in the works of Cai et al.[31b, 31c] |
| 2007 | Cardiology | Intravenously injected $CeO_2$NPs in a transgenic murine model of cardiomyopathy reduced the myocardial oxidative stress, the endoplasmic reticulum stress and suppress the inflammatory process, conferring protection against progression of cardiac dysfunction.[34] |
| 2009 | Chronic inflammation | In vivo study show $CeO_2$NPs potential to reduce ROS production in mice states of inflammation and hence proposed as a novel therapy for chronic inflammation.[15a] |
| 2011 | Diabetes | A combination of $CeO_2$NPs and sodium selenium was beneficial to diabetic rats.[35a] Another, more recent, work can be found e.g. in Khurana et al.[35b] |
| 2013 | Hepatology | CeO2NPs showed similar performance as N-acetyl cystine, a common therapeutic to reduce oxidative stress, in mice with induced liver toxicity (by $CCl_4$).[72] Other, more recent works can be found e.g. in Adebayo et al.[37b] and Fernandez-Varo et al.[37c] |
| 2014 | Regenerative Medicine and tissue engineering | The capacities of $CeO_2$NPs to achieve functional restoration of tissue or cells damaged through disease, aging, or trauma through enhancing long-term cell survival, enabling cell migration and proliferation, and promoting stem cell differentiation were reviewed in the work of Das et al.[39] Another more recent work can be found e.g. in Marino et al.[40] |
| 2017-2019 | NPs in the space | $CeO_2$NPs to counteract the detrimental effects of microgravity-induced oxidative stress.[41] |

[a)]*To the best of our knowledge, we briefly describe here the firsts reports, and more recent ones, that apply the therapeutic potential of $CeO_2$NPs in nanomedicine research. We apologize in advance if other contributions were before the ones here listed as the first one.*

## Table 2.

**Table 2.** Studies showing the therapeutic efficacy of $CeO_2$NPs in different in vitro and in vivo models of liver injury or disease.

| Type of study | Model | Liver injury/disease | $CeO_2$NPs (size, dose, and administration route) | Reference |
|---|---|---|---|---|
| *In vitro* | HepG2 cells (human hepatic cells) | Oxidative stress ($H_2O_2$) | 4 nm; 100 µg/ml | [37a] |
| *In vitro* | HepG2 cells (human hepatic cells) | Hyperglycemia | 8.5 µg/ml | [96] |
| *In vitro* | RAW264.7 cells (murine macrophages) | Lipopolysaccharide | 4-5 nm; 5-1000 µg/ml | [97] |
| *In vitro* | WRL-68 (human hepatocytes) | Oxidative stress (3-Amino-1,2,4-Triazole) | 1.9 nm; 5-200 µmol/L | [98] |
| *In vitro* | Primary portal endothelial cells | Cirrhotic rats | 4 nm; 1 µg/ml | [18c] |
| *In vitro* | HepG2 cells (human hepatic cells) | Steatosis | 4nm; 10 µg/ml | [79] |
| *In vivo* | Sprague Dawley rats | Hepatotoxicity (Monocrotaline) | 25 nm; 0.01 µg/kg; i.p. | [107] |
| *In vivo* | BALB/c-mice | Hepatotoxicity ($CCl_4$) | 3-5 nm; 0.5 mg/kg; i.p. | [15a, 72] |
| *In vivo* | Albino Wistar rats | Hepatotoxicity (D-galactosamine and lipopolysaccharide) | 25 nm; 0.01 µg/kg; i.p. | [105a] |
| *In vivo* | Sprague Dawley rats | Peritonitis (polymicrobial) | 10-30 nm; 0.5 mg/kg; i.v. | [109] |
| *In vivo* | Wistar rats | Liver fibrosis ($CCl_4$) | 4-20 nm; 0.1 mg/kg; i.v. | [37a] |
| *In vivo* | Wistar rats | Cirrhosis ($CCl_4$) | 4 nm; 0.1 mg/kg: i.v. | [18c] |
| *In vivo* | Wistar rats | Non alcoholic fatty liver disease (MCD diet) | 4 nm; 0.1 mg/kg; i.v. | [110] |
| *In vivo* | Wistar rats | Liver regeneration (acetaminophen) | 4 nm; 0.1 mg/kg; i.v | [111] |
| *In vivo* | Wistar rats | Liver regeneration (hepatectomy) | 4 nm; 0.1 mg/kg; i.v | [112] |
| *In vivo* | Wistar rats | Non alcoholic fatty liver disease (Neonatal monosodium glutamate) | 1-5 nm; 1 mg/kg; oral | [113] |
| *In vivo* | Sprague Dawley rats | Hepatic ischemia reperfusion injury | 10-30 nm;0.5 mg/kg; i.v. | [109] |
| *In vivo* | Sprague Dawley rats | Sepsis (Lipopolysaccharide) | 4-5 nm; 0.5 mg/kg; i.v | [97] |
| *In vivo* | Sprague Dawley rats | Hepatotoxicity (Doxorubicin) | 100 nm; 0.5 mg/kg; i.p. | [105b] |
| *In vivo* | BALB/c-mice | Hepatocellular carcinoma (DEN) | <10 nm; 100-200 µg/kg; i.p. | [37b] |
| *In vivo* | Wistar rats | Hepatocellular carcinoma (DEN) | 4-20 nm; 0.1 mg/kg; i.v | [37c] |

## Table 3

**Table 3.** Summary of the advantages of $CeO_2$NPs respect classic antioxidants.

| Classic antioxidants | $CeO_2$NPs |
|---|---|
| No targeted activity. | It can be functionalized, controlled biodistribution. |
| Limited activity: often scavenge one free radical. | Multienzymatic: catalase-like, SOD-like, peroxidase-like activities, NO scavenging, etc. and can participate in the multiplicity of cross-reactions between ROS and inflammation. |
| Limited activity: they are metabolized; after reaction become inactivated. | Not entirely consumed during reaction and thus can work at low doses. |
| Limited activity: short half-life. | Long residence time in tissue. |
| No controlled activity (they become inactivated after reaction). | ROS buffers: only act in conditions of ROS overproduction. |
| Safe | Safe (degraded in innocuous $Ce^{3+}$ ions and expulsed from the body). |

((**For Essays, Feature Articles, Progress Reports, and Reviews,** please insert up to three author biographies and photographs here, max. 100 words each))

Author Photograph(s) ((40 mm broad, 50 mm high, gray scale))

Eudald Casals has been appointed since January 2019 as Head of Laboratory and Distinguished Professor at the Wuyi University (Jiangmen, China). He holds PhD from the Autonomous University of Barcelona (2012). He did several postgraduate stays at the Abo Akademi University (Finland), Vall D'Hebron Research Institute (Spain), European Synchrotron Radiation Facility (France) and the College of Bioengineering (Chongqing University, China). Research interests include the synthesis, conjugation, and characterization of new inorganic nanoparticles for medical applications, renewable energies, environmental remediation, and nanosafety studies.

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## Cerium Oxide Nanoparticles: Advances in Biodistribution, Toxicity and Preclinical Exploration

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The burgeoning potential of antioxidant nanoparticles in theranostic applications faces apparently contradictory reports of either medical benefits or toxicity. This delays translation to clinical practice. Sources of those discrepancies are summarized focusing on the paradigmatic case of cerium oxide in liver disease. This analysis contributes to overcoming similar discrepancies of other nanoparticles and will enable the design of improved nanomedicines.

**Keyword:** cerium oxide nanoparticles, antioxidant nanoparticles, nanomedicine, pharmacokinetics of nanoparticles, liver diseases