**Elucidating the Mechanisms for Plant Uptake and *in-planta* Speciation of Cerium in
Radish (*Raphanus sativus* L.) Treated with Cerium Oxide Nanoparticles**

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Graphical abstract

CeO_2NPs dissolution was enhanced by the low molecular weight organic acids in root exudates and were taken up by radish as both nanoparticles and dissolved ions.

Abstract

Even though the plant uptake of cerium oxide nanoparticles (CeO₂ NPs) has been reported, the mechanisms remain unknown. This study aimed to provide new insights into CeO₂ NPs plant uptake through two objectives: (1) to investigate whether CeO₂ NPs dissolve before their plant uptake and (2) to determine the *in-planta speciation* of Ce. Bench scale experiments were conducted by growing radish in solutions containing 10 mg elemental Ce/L of bulk CeO₂ particles, CeO₂ NPs or ionic Ce. Transmission electron microscope and inductively coupled plasma-mass spectrometry (ICP-MS) analysis suggested that one pathway for CeO₂ NPs uptake was through direct uptake of intact CeO₂ NPs. More importantly, our results confirmed that part of the particulate CeO₂ was transformed into ionic Ce on the root surface before they were taken up by plants. Ionic Ce uptake and transport was a primary mechanism for Ce accumulation in plant shoots. This study further demonstrated that enhanced CeO₂ dissolution on root surface was due to the organic acids with lower molecular weight (e.g. succinic acid) in radish root exudates. Large particles composed of high contents of P and Ce were detected in radish roots treated only with ionic Ce, suggesting the formation of CePO₄ particles. In summary, the results indicated that CeO₂ NPs were taken up by radish as both intact nanoparticles and dissolved ions. Inside plant tissues, Ce is present as a cocktail of CeO₂ NPs, dissolved Ce and Ce salt (e.g. CePO₄) and the specific combination of Ce species is tissue dependent.

Keywords: cerium oxide nanoparticles, radish, transformation, enzymatic digestion, root exudates.

Introduction

It is well recognized that the release of cerium oxide nanoparticles (CeO_2 NPs) into the environment could pose serious risks to human health due to their potential accumulation in food crops [1, 2]. Previous investigations have shown that CeO_2 NPs could be taken up by radish (*Raphanus sativus* L) and many other agricultural crops [3-8] and the health risks associated with the foodborne Ce depend on the physicochemical properties of Ce in plant tissues [9, 10]. A few previous studies reported that Ce in plant tissues remained as CeO_2 NPs after plant uptake [11, 12]. However, several recent publications suggest that Ce accumulated in plant tissues following plant treatment with CeO_2 NPs could exist in the form of Ce salts [13] or dissolved Ce [14]. Due to the importance of the physicochemical states of Ce to its food safety implications, it is important to further understand the physicochemical properties of Ce in plant tissues after plant exposure to CeO_2 NPs.

CeO_2 is generally deemed as an insoluble compound that has stable chemical properties in the environment [15]. However, dissolution does occur under certain circumstances. For example, Schwabe *et al.* [16] found that reductants in plant growth media could adsorb on CeO_2 NP surfaces and reduce the surface Ce^{4+} into Ce^{3+} . Subsequent studies by these researchers further showed that the presence of plants in the growth media and plant root activities are critical for the dissolution and plant uptake of CeO_2 NPs, especially for nanoparticles larger than 20 nm [17]. Zhang *et al.* [13] detected CePO_4 in cucumber roots and cerium carboxylates in cucumber shoots after investigating the chemical valence state of Ce using near edge X-ray adsorption fine structure (XANES) and X-ray scanning transmission microscopy (STXM). A subsequent study by this research group concluded that CeO_2 NPs transformation occurred on the root surface of cucumber, not in plant tissues and stated that root exudates at the nanobio

interface was the necessary condition for the CeO₂ NPs transformation, however, the possible role of plant exudates in CeO₂ NPs dissolution was not explored [18]. Gui *et al.* [19] also found that Ce in lettuce roots treated with 50 – 1000 mg/kg CeO₂ NPs in potting soil presented a mixed oxidation states of Ce³⁺ and Ce⁴⁺. Because CePO₄ and Ce carboxylates were detected in lettuce roots but not in soil, the authors interpreted the results as evidence of *in-planta* transformation of CeO₂ NPs. More recently, Dan et al. [14] investigated the uptake of CeO₂ NPs by four different plant species using a highly sensitive single particle inductively coupled plasma mass spectrometry (SP-ICP-MS) technology and reported common presence of dissolved Ce in plant tissues. In spite of these new progresses, questions remain concerning the mechanisms for CeO₂ NPs uptake by plant, the location of Ce transformation (*e.g.* within plant tissues or in the environment) and the composition of Ce in plant tissues following their uptake. Therefore, it is critical to further investigate the physicochemical properties of Ce in plant tissues and in the surrounding environment of plants during CeO₂ NPs uptake to gain more insights into the plant uptake of CeO₂ NPs and their transformation.

While strong acid digestion is commonly used in CeO₂ NPs studies, the unique features of CeO₂ NPs were lost during the digestion process. A key challenge to study the physicochemical properties of Ce in plant tissues is the development of an extraction method which could effectively extract Ce from plant tissues without altering its properties. Enzymatic digestion by proteases or pectinases has been successfully used to solubilize biological materials and extract engineered nanoparticles (ENPs) [20-24]. A recent breakthrough in extracting ENPs from plant tissues with Macerozyme R-10 provides a unique opportunity to elucidate the uptake mechanisms of CeO₂ NPs by plants and to determine the chemical properties of Ce in plant tissues. To date, gold nanoparticles (Au NPs) and CeO₂ NPs have been successfully extracted

from plant tissues using the above-mentioned enzymatic method. In this study, the same enzymatic extraction method with Macerozyme R-10 was adopted. The objectives of this study were (1) to determine the physicochemical properties of Ce in radish following plant exposure to different types of Ce; and (2) to further investigate the mechanisms for CeO₂ NPs plant uptake and *in-planta* speciation. Radish was chosen as a model plant for several reasons: (1) its global popularity; (2) its easiness to grow and (3) its strong relevance to food safety due to the direct contact of its edible tissue with environmental pollutants.

Materials and Methods

Ce preparation and characterization

Dispersion of bare CeO₂ NPs (10 wt % in H₂O) and CeCl₃·7H₂O crystals were purchased from Sigma-Aldrich (St. Louis, MO, USA). The bulk powder of CeO₂ was obtained from Strem Chemicals (Newburyport, MA, USA). Four treatments were prepared: (1) control (no Ce treatment); (2) 10 mg Ce/L of CeO₂ NPs dispersion (NPs); (3) 10 mg Ce/L of bulk CeO₂ suspension (bulk); and (4) 10 mg Ce/L of CeCl₃ solution (ion). Dispersion of bare CeO₂ NPs was filtered through a 10 kDa Amicon Ultra-4 Centrifugal Filter (EMD Millipore, Billerica, MA, USA) unit to remove dissolved Ce (if present) in the dispersion. The bulk CeO₂ was dispersed in deionized water and filtered through a P8 grade filter paper (Fisherbrand, Pittsburgh, PA, USA) to remove nanosized particles. The CeO₂ NPs and bulk CeO₂ residue on the filter was air-dried at room temperature. The dried CeO₂ NPs and bulk CeO₂ were then re-dispersed in water by sonication. The newly obtained dispersions were diluted to obtain suspensions of the targeted concentration. The size and morphology of CeO₂ NPs and bulk CeO₂ were determined by a Tecnai G2 F20 transmission electron microscope (TEM) (FEI, Hillsboro, OR, USA) and a JEOL 6400 scanning electron microscope (SEM) (JEOL USA, Inc. Peabody, MA, USA) respectively.

The TEM image of CeO₂ NPs, SEM image of bulk CeO₂, and their corresponding particle size distributions are shown in **Fig. S1**. Most CeO₂ NPs fell in the size range of 10-40 nm, with an average size of 15.51 nm. The average particle size was obtained by measuring the diameter of over 100 individual nanoparticles with an imaging processing software ImageJ (ver 1.49). Bulk CeO₂ particles had a broader size distribution ranging from 2 to 13 μ m, with an average size of 6.2 μ m.

Plant cultivation and harvest

The radish seeds [Cherriette (F1)] were purchased from Johnny's Selected Seeds (Winslow, ME, USA). The procedures for seed germination and seedling development followed the established protocols in our previous research [25]. Briefly, the sterilized seeds were germinated on a moist filter paper in a Petri dish for five days. Healthy young seedlings were then transferred to 50 mL polypropylene centrifuge tubes containing quarter strength Hoagland solution (Phytotechnology Laboratories, Lenexa, KS, USA). Plants were incubated in a growth cart with a 16-hour light/8-hour dark cycle (28 °C) to allow the seedlings to further develop. After two weeks, the seedlings were transferred to the prepared Ce suspension/solution for 5 days. Each treatment had three replicates. The suspension/solution in the tubes was replenished daily with the same suspension/solution to compensate for transpiration. The daily water consumption of each plant was recorded. The plants were sacrificed at day 24 after germination.

At harvest, plants were separated into three sections: fine roots, storage root, and shoots (**Fig. S2**). The storage root and fine roots were washed with 50 mL CaCl₂ solution (5 mM) five times to remove the adhering Ce on the tissue surfaces. To determine the washing efficiency of CaCl₂, plant roots collected from an individual plant were evenly divided into two groups, with one rinsed with DI water only and the other one rinsed with 5 mM CaCl₂ solution five times. The

roots were then acid digested and the total Ce in plant root tissues quantified with an ICP-MS. The study was replicated three times and the average removal efficiency of Ce by CaCl_2 was over 95%. The unwashed and washed fine roots were also observed under a Tescan Vega 3 scanning electron microscope (SEM) (Tescan USA Inc. Warrendale, PA, USA) to confirm the washing effectiveness (**Fig. S3**). The SEM images indicated that the majority of Ce deposit on root surface was removed by washing. The washing solutions and the growth media were collected and filtered through 10 kDa Amicon Ultra-4 Centrifugal Filter units to separate the dissolved Ce from the particulate Ce. The concentrations of dissolved Ce in the filtrates of washing solutions and growth media were determined by an Elan DRC II ICP-MS (Perkin Elmer, Waltham, MA, USA). A fraction of the washing solution and the growth media was also acid digested to determine the total Ce in them. The acid digestion was performed using a DigiPREP MS hot block digester (SCP science, Clark Graham, Canada). 5 mL of 70% (w/w) nitric acid (Certified ACS Plus) was added to 1 mL of the washing solution or the growth medium and heated at 95 °C for 4 hours. After cooling down to room temperature, 3 mL of 30% (w/w) H_2O_2 (Certified ACS) was added to the mixture and heated in the hot block at 95 °C for another 2 hours to completely digest the sample. The Ce was then quantified by an ICP-MS as the total Ce in the washing solution or the growth medium after proper dilution.

Enzymatic extraction, acid digestion, and ICP-MS detection of Ce from plant tissues

The enzymatic extraction of Ce from plant tissues was conducted following the recently developed procedure⁹. The washed tissues were cut into small pieces with a blade, from which 0.5 g was weighed and mixed with 9 mL of 20 mM 2-(N-morpholino)ethanesulfonic acid (MES) buffer (pH=5, adjusted by NaOH). The mixture was homogenized in a centrifuge tube using a handheld homogenizer. One mL of 30 mg/mL Macerozyme R-10 enzyme (prepared in 20 mM

MES) was then added into the mixture. The mixture (10 mL) was shaken at 37 °C for 24 hours. Afterwards, 4 mL of the digestate was filtered through a 10 kDa Amicon Ultra-4 Centrifugal Filter unit. The Ce in the filtrate was quantified by ICP-MS as the dissolved Ce in plant tissues. Another 4 mL aliquot of enzyme digestate was acid digested to determine total Ce in plant tissues following the same protocol mentioned above.

TEM imaging of the enzymatic digestate

Drops of the last 2 mL of remaining enzymatic digestate were air-dried on a TEM copper grid and then observed under a Tecnai G2 F20 TEM equipped with an energy dispersive spectroscopy (EDS) at 200 kV to examine the physical and chemical form of Ce microscopically.

Artificial root exudates (ARE) study

The simplified ARE contained a mix of low molecular weight organics previously reported to occur in plant root exudates: glucose (50 mM), succinic acid (25 mM), malic acid (25 mM), and serine (12.5 mM) [26-28]. The simplified ARE and the four individual components at the same concentration as in the ARE were mixed with 10 mg/L CeO₂ NPs dispersion in a centrifuge tube. The CeO₂ NPs dispersion at 10 mg/L in DI water was prepared as a reference. The mixtures were shaken on a shaker for 5 days to simulate the Ce exposure process of radish. Afterwards, the mixtures were filtered through 10 kDa Amicon Ultra-4 Centrifugal Filter units. The dissolved Ce in the filtrate was measured by ICP-MS. The total Ce in the mixtures were determined by acid digestion as mentioned above, followed by ICP-MS. The ratios of dissolved Ce to total Ce in the mixtures were then calculated.

Data Analysis

The results are reported as mean $\pm$ standard error of three replicates. One-way ANOVA analysis and Duncan's test for post hoc comparisons were performed with IBM SPSS Statistics V22.0. Different letters in the Figures represent significant differences among the treatments when the p value was <0.05 .

Results and Discussion

The fresh biomass of different radish tissues was shown in **Fig. S2**. The results were consistent with our previous observation that CeO₂ NPs at the tested concentration enhanced the growth of radish storage roots [5], while the ionic Ce³⁺ at the same concentration inhibited radish growth [4].

The concentrations of Ce in plant tissues were shown in **Fig. 1**. The total Ce concentration was highest in plant roots exposed to Ce³⁺ ions, followed by the plants exposed to CeO₂ NPs, which was significantly lower than Ce³⁺ treated plants but significantly higher than bulk CeO₂ treated plants or the controls. Ce concentrations in radish roots exposed to the bulk CeO₂ were not statistically different from the controls. In radish shoot tissues, only ionic Ce treated plants had significantly higher total Ce concentration. The remaining three treatments did not cause any significant differences in the total Ce in radish shoots ($P>0.05$ in one-way ANOVA analysis). Similar to the total Ce concentrations in plant tissues, exposure to Ce³⁺ ions resulted in significantly higher concentrations of dissolved Ce in all radish tissues compared with the control plants. No dissolved Ce was detected in the storage root and fine roots of the control radish. Dissolved Ce concentrations detected in tissues treated with bulk CeO₂ and CeO₂ NPs were significantly lower than those detected in plant tissues exposed to ionic Ce³⁺. The

concentrations of dissolved Ce in the storage root and fine roots of bulk CeO₂ treated radish were significantly lower than those treated with CeO₂ NPs, indicating the significant impact of particle size on the release of dissolved Ce from CeO₂ particles. Intact CeO₂ NPs, which had similar particle size distribution to the primary CeO₂ NPs dosed in the growth medium (**Fig. S1a**), were detected in the unfiltered enzymatic digestate of fine roots exposed to CeO₂ NPs (**Fig. 2a**). These particles could come from either the residues of CeO₂ NPs deposit on root surface or the nanoparticles penetrated into radish fine roots. Because several previous studies including our own studies have demonstrated the uptake of CeO₂ NPs by crop roots visualized by confocal microscope, TEM or μ -XRF [12, 29, 30], we conclude that at least part of the detected CeO₂ NPs in the fine root digestate have penetrated into the root tissues. However, no CeO₂ NPs were found in the enzymatic digestates of shoots and storage root of radishes exposed to CeO₂ NPs (**Fig. S4**), indicating that the upward transport of CeO₂ NPs in radish was limited. The results suggested that the nanoparticles within the fine root tissues were most likely in the epidermis and cortex tissues, without reaching to the vascular tissues. Due to the detection of CeO₂ NPs in plant fine roots and the dissolved Ce in all radish tissues treated with CeO₂ NPs alone, there are two possibilities for the origin of the dissolved Ce: (1) CeO₂ dissolution within the fine roots after they entered into the root tissue or (2) part of the CeO₂ NPs dissolve in the rhizosphere and both dissolved Ce and CeO₂ NPs were taken up by plant fine roots, but only the dissolved Ce was transported from roots to shoots. Based on the concentrations of dissolved Ce and the ratios of dissolved Ce to the total Ce in the growth media and washing solution at harvest (**Table 1**), dissolution of CeO₂ NPs and bulk CeO₂ in the growth media was minimal. The results agreed well with the data in the artificial root exudates study that CeO₂ NPs dissolution in water without plants was insignificant after 5 days (first column in **Fig. 3**). In contrast, significant amount of

dissolved Ce and much higher ratios of dissolved Ce to total Ce were detected in the washing solutions of radish roots from CeO₂ treated plants. The concentration of dissolved Ce in the washing solution of control plants was negligible. CaCl₂ at 5 mM could reduce the Debye length of CeO₂ NPs and cause CeO₂ NPs aggregation, but they were not expected to cause dissolution of CeO₂ [31]. Thus, the high concentrations of dissolved Ce in the washing solution reflected the higher percentage of dissolved Ce in the rhizosphere or on root surface, strongly indicating the dissolution of Ce from CeO₂ particles before they were taken up by plants. The higher ratio of dissolved Ce to total Ce in the washing solution from CeO₂ NPs treatment also indicated that the location of CeO₂ NPs transformation was the root surface of radish. In addition, the quantitative calculation of the dissolved Ce in the collected washing solutions (250 mL) and plant tissues from CeO₂ NPs treatment showed that the amount of dissolved Ce was highest in the surface deposit, followed by fine roots, storage root and then the shoots. Altogether, our results strongly suggest the dissolution of CeO₂ NPs occurred in the rhizosphere before ionic Ce was taken up by plant roots. This finding agrees well with the results reported by Schwabe et al. [17] and Ma et al. [18] that root surface was the primary site for CeO₂ NPs transformation.

Ma et al. [18] attributed the enhanced dissolution of CeO₂ on the root surface of cucumber to the root exudates. However, the underlying mechanism for the interactions between root exudates and CeO₂ NPs was not elucidated. Plant roots release a wide range of compounds such as, ions, free oxygen, enzymes, mucilage, and a diverse array of carbon-containing molecules [32]. Among them, sugars, organic acids with low molecular weight (MW), and amino acid are the most common compounds in the exudates [26-28]. Consistent with CeO₂ NPs studies, previous research has shown that lettuce root exudates improved the solubilization of TiO₂NPs and Fe₃O₄NPs in the rhizosphere [33]. Stegemeier et al. also found that the root

exudates of Alfalfa (*Medicago sativa* L.) could partially dissolve Ag₂S-NPs, making them more bioavailable to plants [34]. In this *in vitro* study, we showed for the first time that the enhanced dissolution of CeO₂ NPs is primarily due to the organic acids with low molecular weight in root exudates (e.g. succinic acid and malic acid) (**Fig. 3**). Due to the small pore sizes in plant roots which could physically prevent the entrance of CeO₂ NPs into plant root tissues, we postulate that CeO₂ NPs dissolution caused by the organic acids with low molecular weight in root exudates is a key mechanism for CeO₂ NPs uptake and accumulation, especially in plant shoots. Interestingly, the malic acid caused significantly higher CeO₂ NPs dissolution than the succinic acid. Malic acid has higher acidity (pKa1=3.4) than succinic acid (pKa1=4.2) [35], implying that low pH resulted from the addition of these acids might be an important factor governing the CeO₂ NPs transformation. This finding was consistent with a previous report that the percentage of Ce³⁺ ions released from CeO₂ NPs (20 nm) increased from 0.25% to 3.1% as pH decreased from 7 to 4 in soil [36]. Future research is needed to better understand the mechanisms for enhanced dissolution of CeO₂NPs on root surface by root exudates. For example, it is still unclear whether the root exudates facilitated CeO₂ NPs transformation on root surface is a redox reaction or dissolution process due to the lower pH levels on root surface. It is worth noting that root exudates for a plant species change with age. Therefore, the extent of CeO₂ NPs dissolution and their uptake and accumulation in plants may vary depending on the growth stages of plants when they were first exposed to CeO₂ NPs. The results from this study may partially explain our previous observation that the phyto-impact of CeO₂ NPs differ at different plant growth stages.

The concentrations of total Ce in all treated radish tissues were significantly higher than the concentrations of dissolved Ce in the corresponding tissues, implying the existence of undissolved Ce within plant tissues. Dan et al. [14] detected particulate Ce signals in plant

tissues spiked with dissolved Ce only using the same enzymatic extraction method adopted in this study. The authors attributed it to the attachment of dissolved Ce on the undigested plant tissue colloids so that the SP (single particle)-ICP-MS mistakenly reported this adsorbed Ce as particulate Ce. Therefore, the concentration of measured dissolved Ce was probably lower than the actual concentration of dissolved Ce within plant tissues. Another potential reason was the re-formation of particulate Ce in addition to direct CeO₂ NPs uptake. CePO₄ was reported as an undissolved form of Ce following the Ce³⁺ uptake process [13, 19]. Our results also suggested the formation of CePO₄ in radish fine roots and storage root. For example, large particles were found in the digestates of storage root and fine roots of radish exposed to ionic Ce³⁺ only (**Fig. 2 b, c**). An X-ray analysis on the elemental composition of the particles indicated higher contents of P in addition to Ce, suggesting that the particles might be in the form of CePO₄. If CeO₂ NPs were reduced to Ce³⁺ in the rhizosphere and Ce³⁺ was taken up by plants and formed insoluble CePO₄, then Ce in plant tissues could be present as a mixture of intact CeO₂ NPs, dissolved Ce, and Ce salts after plants are exposed to CeO₂ NPs. Due to the low transport potential of intact CeO₂ NPs from roots to other tissues, the specific composition of Ce and their relative abundance may be tissue dependent.

In conclusion, the results of this study demonstrated the transformation of CeO₂ NPs to dissolved Ce on the root surface of radish and this transformation provides a significant mechanism for plant uptake of CeO₂ NPs. We further confirmed that organic acids with low molecular weight in root exudates are key factors for enhanced CeO₂ NPs dissolution in plant rhizosphere, even though the chemical principles for root exudates enhanced dissolution are still unknown. The dissolved Ce and part of the CeO₂ NPs could be directly taken up by radish fine roots. Particulate Ce (e.g. CePO₄) could be formed after ionic Ce was taken up by plants in plant

tissues. As a result, Ce in plant tissues could be present as a mixture of CeO₂ NPs, dissolved Ce, and Ce salts and the specific composition and relative abundance of these different species of Ce is tissue dependent. The co-existence of various forms of Ce complicates the health implications of Ce in plants and this phenomenon needs to be considered when the potential human exposure to Ce through plant consumption is investigated.

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Fig. 1. Average Ce concentration in radish tissues (n=3). The error bars represent standard error of the mean. Different letters above the bars represent significant differences among the treatments ($P < 0.05$) as determined by One-way ANOVA analysis followed by Duncan's test for post hoc comparisons.

Fig. 2. TEM images of (a) the enzymatic digestate of fine roots exposed to CeO_2 NPs, (b) the enzymatic digestate of storage root exposed to Ce^{3+} ions, and (c) the enzymatic digestate of fine roots exposed to Ce^{3+} ions. The EDS spectrums under the TEM images show the detected elements in selected areas (red frames in images).

Fig. 3. The ratios of dissolved Ce to total Ce in the reference (CeO₂ NPs dispersion only) and in the mixtures of CeO₂ NPs and artificial root exudates. Different letters above the bars represent significant differences among different treatments ($P < 0.05$) as determined by One-way ANOVA analysis followed by Duncan's test for post hoc comparisons.

Table 1. Concentrations of dissolved cerium and the ratio of dissolved Ce to total Ce in the growth media and washing solution at harvest (mean $\pm$ standard error, n=3).

Dissolved Ce	Control	Bulk	NPs	Ion
Washing solution ($\mu\text{g/L}$)	0.16 $\pm$ 0.08	0.51 $\pm$ 0.12	23.03 $\pm$ 2.42	414.33 $\pm$ 54.15
Growth media ($\mu\text{g/L}$)	0.22 $\pm$ 0.11	0.45 $\pm$ 0.18	0.21 $\pm$ 0.14	2049.33 $\pm$ 425.34
Dissolved Ce / Total Ce	Control	Bulk	NPs	Ion
Washing solution (%)	-	0.24 $\pm$ 0.02	4.21 $\pm$ 0.55	-
Growth medium (%)	-	0.02 $\pm$ 0.00	0.024 $\pm$ 0.01	-