The potentiation effect makes the difference: Non-toxic concentrations of ZnO nanoparticles enhance Cu nanoparticle toxicity *in vitro*

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HIGHLIGHTS

- ZnONPs at non-toxic concentrations increased the toxicity of CuNPs *in vitro*.
- ZnONPs of larger size provoked a stronger synergistic effect with CuNPs.
- The synergistic effect was attributed to the particle fraction of ZnONPs.

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ABSTRACT

Here we examined whether the addition of a non-toxic concentration (6.25 µg/mL) of zinc oxide nanoparticles (ZnONPs: 19, 35 and 57 nm, respectively) modulates the cytotoxicity of copper nanoparticles (CuNPs, 63 nm in size) in the human hepatoma cell line HepG2. The cytotoxic effect of CuNPs on HepG2 cells was markedly enhanced by the ZnONPs, the largest ZnONPs causing the highest increase in toxicity. However, CuNPs cytotoxicity was not affected by co-incubation with medium containing only zinc ions, indicating the increase in toxicity might be attributed to the particle form of ZnONPs. Transmission electron microscopy (TEM) revealed the presence of CuNPs and ZnONPs inside the cells co-exposed to both types of NP and outflow of cytoplasm through the damaged cell membrane. Inductively coupled plasma mass spectrometry (ICP-MS) determined an increase in the concentration of zinc and a decrease in that of copper in co-exposed cells. On the basis of these results, we propose that accumulation of large numbers of ZnONPs in the cells alters cellular membranes and the cytotoxicity of CuNPs is increased.

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1. Introduction

Over the last two decades, a large number of engineered metal-containing nanomaterials with varying particle sizes have covertly entered our daily lives. According to the Nanotechnology White Paper published by the US-EPA, metal oxides are the most widely used engineered nanomaterials (U.S. EPA, 2007). Among the various engineered nanomaterials, zinc oxide nanoparticles (ZnONPs) are widely used all over the world (Gottschalk et al., 2009). Consequently, ZnONPs have inevitably entered the milieu, thus raising concerns about the potential threats they pose to the environment and public health. Copper nanoparticles (CuNPs) are commonly used as superconductors and catalysts. They are applied to lithium ion electrode

materials, bioactive coatings, and liquid films. Given such wide use of these NPs, there is a high risk of their release into the environment and of exposure to humans and wildlife.

The divalent ion forms of Zn and Cu are nutritionally essential for all living organisms as they are cofactors for a number of enzymes. However, when present in excess, these metals can cause adverse effects (Regoli and Principato, 1995; Wilson et al., 2012). The toxicity of zinc and copper has long been recognized, and a number of studies have already addressed issues related to the bioavailability, bioaccumulation, and deleterious activities of soluble forms of these two metals (Babich and Stotzky, 1978; Jonas, 1989). The mutual interactions of zinc and copper at the nutritional level are also well known. These metals mutually inhibit absorption in the intestine (Hall et al., 1979; Hogstrand, 2011). This effect is caused by feed-back mechanisms affecting metal regulatory proteins. For instance, an increase in cellular copper can lead to the down-regulation of zinc importer proteins and channels

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(Qiu and Hogstrand, 2005) and the up-regulation of metallothionein as a zinc buffering protein (Westin and Schaffner, 1988). Interestingly, inhibitory effects of zinc on copper uptake in the intestine have also been observed (Wapnir and Balkman, 1991).

Although the possible deleterious effects of high or low concentrations of these metals are recognized, there is a considerable knowledge gap about the toxic effects of nanoparticles (NPs) whose structure is based on zinc or copper (Li et al., 2014; Ma et al., 2014). In order to shed light on this field, we recently performed in-depth studies in a variety of hepatic cell lines of mammalian and piscine origin in an attempt to determine the contribution of ionic and NP fractions to the cytotoxicity of ZnONPs and CuNPs (Fernandez-Cruz et al., 2013; Song et al., 2014). We revealed the complexity of the toxic effects of these nanomaterials and showed that although the toxicity is influenced by the metal (Zn or Cu) ions released during the dissolution of NPs in the culture medium, the NP itself greatly contributes to the toxicity observed.

Organisms are exposed to soluble zinc and copper ions in the natural environment. In this regard, these previous studies raised the question about the possible interaction between ZnONPs and CuNPs in the environment and about the mutual influence of these materials on their toxicity. Indeed, the simultaneous presence of both kinds of nanomaterials in the environment has already been reported, for instance in wastewater effluents (Bolyard et al., 2013; Gysztzejska-Piotrowaka et al., 2009), thus raising considerable concerns about the potential adverse effects of co-exposure. Interactions between these materials may occur at the NP level, but NPs of each material may also influence the processes or toxicity caused by the ions released by the other. A number of studies have addressed the possible modulation of the activity of ions or chemicals by NPs. For instance, Tan et al. (2012) showed that TiO₂NPs enhance the uptake and retention of zinc and cadmium in *Daphnia magna*, and Torre-Roche et al. (2013) demonstrated that exposure of *Cucurbita pepo* (zucchini) and *Glycine max* (soybean) to silver nanoparticles (AgNPs) leads to a significant reduction in the uptake of *p,p'*-DDE. However, with some exceptions (Guo et al., 2009; Tang et al., 2013), little attention has been given to the effects of co-exposure to ZnONPs and CuNPs.

Here we sought to study the mutual influence of ZnONPs and CuNPs on the toxicity of these particles *in vitro* using a human hepatoma cell line, HepG2. The data obtained here will serve to set the basis for more realistic and complex experiments *in vivo*.

2. Materials and methods

2.1. Chemicals

We used one type of CuNPs (50 nm according to the manufacturer, IoliTec, Inc., Germany) and three types of ZnONPs (with sizes of 25, 50 and 100 nm according to the manufacturer, Sigma-Aldrich, Madrid, Spain). All particles were uncoated. With the exceptions indicated below, all chemicals were supplied by Sigma-Aldrich and all the products for cell culture by Lonza (Barcelona, Spain). A detailed description of the materials and chemicals is included in the Supplementary Material.

2.2. Cell culture and exposure

The HepG2 cell line was obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). Cells (5×10^5 cells/mL) were cultured in Eagle's minimum essential medium (EMEM) supplemented with 10% fetal bovine serum (FBS), 1% penicillin/streptomycin (P/S), 1% ultraglutamine and 1% non-essential amino acids 100× (NEAA) and exposed to NPs in 96-well plates (Greiner Bio-one GmbH) following the method previously described (Fernandez-Cruz et al., 2013).

Stock CuNPs and ZnONPs suspensions of 200 µg/mL were freshly prepared and dispersed in serum-free medium using sonication for

10 min in an S 40H Elmasonic water bath sonicator (Elma, Germany). Afterwards, vortexing for 1 min was used before applying suspensions to the cells, as described in Song et al. (2014) and Fernandez-Cruz et al. (2013) respectively. A volume of 100 µL of NP suspension in serum-free medium was added to each well. Final concentrations of CuNPs ranging from 0.39 to 25.0 µg/mL were applied alone or together with 6.25 µg/mL ZnONPs (a non-toxic concentration for HepG2 cells, as determined by Fernandez-Cruz et al., 2013). For NP co-exposure, CuNPs and ZnONPs suspensions were first mixed (v/v = 1:1) and then added to the culture wells. As a control, cells exposed to serum-free medium were used. Exposure lasted for 48 h, after which the medium was removed, and three cytotoxicity assays were performed.

To establish the contribution of Zn ions to the toxicity caused by ZnONPs, cells were also co-exposed to CuNPs and to supernatants obtained after centrifugation (5500 rpm for 2 h at 4 °C) of ZnONPs suspensions. A Lince R centrifuge (Orto Alresa, Spain) was used for this purpose.

2.3. Cytotoxicity assays and protein measurements

We checked for possible interference of NPs with the wavelengths used to perform the measurements in the assays. No interaction at any of the concentrations used was detected.

The 4,5-dimethylthiazolyl-(2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, which evaluates effects on mitochondrial activity, was done following the method described by Mosmann (1983), which is based on the enzymatic conversion of MTT salts to formazan crystals. After removal of the medium, the cells were washed twice with PBS, and 100 µL of phenol red-free MEM containing MTT (0.5 mg/mL) was added to each well. The plates were incubated for 2 h at 37 °C. The formazan crystals were then dissolved by adding 100 µL of isopropanol. Absorbance was measured at 570 nm using a Genios microplate reader (TECAN, Männedorf, CH).

The 6-carboxy-2',7'-dichlorofluorescein diacetate (CFDA) assay was performed following Schirmer et al. (1997). CFDA is an esterase substrate that living cells can convert into a polar fluorescent dye. Its release outside of the cell and consequent reduction of concentration inside the cell is indicative of damage to the plasma membrane. After washing the exposed cells twice with PBS, we added 100 µL of phenol red-free MEM containing CFDA reagent to each well. After a 30-min incubation at 37 °C, fluorescence was measured at an excitation wavelength of 485 nm and an emission wavelength of 530 nm using a TECAN microplate reader.

The neutral red uptake (NRU) assay was done following Borenfreund and Puerner (1985). Since NR is accumulated in functional lysosomes in living cells, this test allows the assessment of lysosomal membrane integrity. After 48 h of exposure, the cell medium was removed, and the cells were washed twice with PBS. Next, 100 µL of phenol red-free MEM containing NR dye (0.1 mg/mL) was added to each well. The plates were incubated for 2 h at 37 °C, and the medium containing the NR dye was removed. The retained NR dye was extracted with 100 µL of 1% glacial acetic acid in 50% ethanol. Absorbance was measured at 550 nm using a TECAN microplate reader.

Total protein content was measured using the fluorescamine method (Udenfriend et al., 1972). Fluorescence was measured using a TECAN microplate reader at excitation/emission wavelengths of 360 and 450 nm.

2.4. Microscopy analyses

Transmission electron microscopy (TEM) was used to determine the size of pristine CuNPs and ZnONPs and of these NPs in suspensions (100 µg/mL) obtained in serum-free EMEM. For TEM analysis of pristine NPs, ethanol was used as a dispersant. A JEOL 2100 HT (JEOL Ltd., Japan) model operating at an accelerating voltage of 200 kV was used. Ultrathin sections of cells were analyzed by TEM to reveal the intracellular

accumulation/distribution of NPs and possible ultrastructural modifications. The method used has been described in Lammel et al. (2013), which is briefly shown in the Supplementary Materials. A JEOL 1010 JEM (JEOL) microscope operating at an acceleration voltage of 100 kV with integrated energy dispersive X-ray (EDX) spectroscopy (Oxford INCA) was used.

2.5. Inductively coupled plasma mass spectrometry

The cellular concentration (we were unable to distinguish between the inside and the surface of the cell) of zinc and copper was determined by inductively coupled plasma mass spectrometry (ICP-MS), as described in Fernandez-Cruz et al. (2013) and in Song et al. (2014). Details of the method have been included in the Supplementary Material. For this analysis, cells were exposed to CuNPs and ZnONPs in 6-well plates (Greiner Bio-one GmbH) and were cultured at an initial density of 1.25×10^6 cells/well in a total volume of 2.5 mL of medium per well. Exposure experiments were done as previously described. After exposure, the culture medium was removed, and cells were rinsed twice with PBS (2.5 mL/well) to remove the NPs remaining in the wells before cell collection for metal analyses.

2.6. Statistical analysis

Results obtained in the co-exposure experiments with each CuNPs concentration and a fixed ZnONPs concentration (6.25 $\mu\text{g/mL}$) were compared with the toxicity results corresponding to cells exposed to the CuNPs alone at the corresponding concentration. The normality and homoscedasticity of all data were checked by the Shapiro–Wilk

test and Bartlett's test, respectively. For comparisons, a parametric repeated measurements-analysis of variance (RM-ANOVA) followed by a Dunnett's post hoc test was applied. The concentration–response function and IC_{50} (Inhibition Concentration 50, defined for the cytotoxicity assays as the concentration causing a 50% inhibition with respect to the maximal response observed in the controls) were estimated by fitting the cytotoxicity results to a regression equation for a sigmoidal curve: $y = \text{max}/[1 + (x / \text{IC}_{50})^b] + \text{min}$, (where max is the maximal response observed, b is the slope of the curve, and min is the minimal response). SigmaPlot 12.0 (Systat Software Inc., Chicago, IL, USA) was used for all the statistical analysis and to calculate the IC_{50} values.

3. Results

3.1. Nanoparticle shape and size

TEM images revealed that the pristine CuNPs were spheroidal (Fig. S1A in the Supplementary Material), with a mean diameter of 63 ± 16 nm. Fig. 1A shows the size distribution histogram obtained from 145 measurements. ZnONPs were trapezoidal with round borders, and the mean sizes of three ZnONPs were 19 ± 4 , 35 ± 11 and 57 ± 16 nm, respectively (Fig. S1B, C and D). The size frequency distribution histograms of pristine ZnONPs ($n = 145$) are shown in Fig. 1B, C and D.

In addition, we further characterized the size of NPs in the serum-free culture medium used for the cytotoxicity test. TEM micrographs revealed that the NPs in the medium showed a higher degree of aggregation than pristine particles (Fig. S2). The frequency size distribution of each NP in culture medium was determined by dynamic light scattering (DLS). Particle sizes of CuNPs and ZnONPs measured by DLS were 265,

Fig. 1. Primary particle size distribution histograms of NPs used in this study. (A) CuNPs (40–93 nm), (B) ZnONPs (13–30 nm), (C) ZnONPs (22–60 nm), and (D) ZnONPs (35–100 nm).

1134, 1260 and 2978 nm respectively, which has been reported in our previous studies (Fernandez-Cruz et al., 2013; Song et al., 2014).

3.2. Effects of a non-toxic concentration of ZnONPs on the cytotoxicity of CuNPs

HepG2 cells exposed to CuNPs alone showed a concentration-dependent drop in viability when measured by the MTT assay, with a

Fig. 2. Data obtained from cytotoxicity assays for CuNPs and ZnONPs. The concentrations of CuNPs ranged from 0.39 to 25.0 μg/mL, and those of ZnONPs were fixed at 6.25 μg/mL. (A) MTT assay, (B) CFDA assay, and (C) NRU assay. Results are expressed as percentage of viability compared with the control cells (i.e. cells without toxicant). Values represent the mean ± standard deviation of at least three independent experiments. Asterisks denote differences with respect to the effect produced by the CuNPs alone at each concentration tested (one-way RM-ANOVA followed by Dunnett's test): * $p < 0.05$, ** $p < 0.01$ and *** $p \leq 0.001$.

marked decrease at concentrations of 12.5 μg/mL or higher (Fig. 2A). None of the ZnONPs caused cytotoxicity in HepG2 cells, thereby indicating that the cells showed a normal mitochondrial function.

Surprisingly, the addition of ZnONPs to the CuNPs caused a clear increase in toxicity. Moreover, we found that the reduction in cell viability was related to the size of the ZnONPs. For the smallest ones (19 nm), significant differences in cell viability ($p < 0.05$) with respect to the cells exposed to CuNPs alone were observed only at the highest CuNPs concentration (25.0 μg/mL), while ZnONPs with a diameter of 35 nm led to a significant ($p < 0.05$) decrease in viability with respect to cells treated with CuNPs alone at a concentration of 12.5 μg/mL. The largest ZnONPs (57 nm, 6.25 μg/mL) caused a significant ($p < 0.05$) increase in toxicity with respect to cells exposed only to CuNPs at a concentration of 0.39 μg/mL (Fig. 2A).

In the CFDA and NRU assays, exposure to CuNPs alone caused a decrease in cell viability only at the highest CuNPs concentration (25.0 μg/mL). The results of these assays also showed enhanced cytotoxicity of CuNPs in HepG2 cells after addition of ZnONPs (Fig. 2B and C). In the CFDA assay, addition of ZnONPs with a diameter of 19 nm led to a significant ($p < 0.05$) decrease in cell viability with respect to cells exposed to CuNPs concentrations of 0.78 μg/mL or higher. ZnONPs with a size of 35 nm caused an increase ($p < 0.001$) in the cytotoxicity produced by a CuNPs concentration of 25.0 μg/mL. Finally, the largest ZnONPs (57 nm) led to a decrease ($p < 0.01$) in cell viability compared to that of the lowest CuNPs concentration tested (0.39 μg/mL).

In the case of the NRU assay, ZnONPs (19 nm) led to an increase ($p < 0.01$) in the cytotoxicity produced by the CuNPs when present at a concentration of 3.12 μg/mL; the ZnONPs (35 nm) caused this effect ($p < 0.001$) at a CuNPs concentration of 12.5 μg/mL; and the largest ZnONPs (57 nm) led to a reduction ($p < 0.001$) in cell viability at a CuNPs concentration of 0.39 μg/mL.

The IC_{50} values corresponding to CuNPs alone and also to co-exposure to CuNPs and ZnONPs of different sizes were calculated for the MTT assay (Fig. 3). The IC_{50} corresponding to the co-exposure to CuNPs and the largest ZnONPs (57 nm) was lower than that calculated after exposure to CuNPs alone or after co-exposure to CuNPs and the smallest ZnONPs (19 or 35 nm).

3.3. Species-specific contribution of ZnONPs to the enhanced cytotoxicity of CuNPs

We used the MTT assay to evaluate and compare the cytotoxicity of CuNPs alone with that of the supernatants obtained after centrifugation of ZnONPs and also with that of the mixtures of ZnONPs supernatants

Fig. 3. IC_{50} calculated after exposure of HepG2 cells to CuNPs alone or to CuNPs (0.39–25.0 μg/mL) plus ZnONPs at a concentration of 6.25 μg/mL, according to the results from the MTT assay.

Fig. 4. (A) Viability of HepG2 cells exposed to ZnONPs supernatants (Sup) and CuNPs, as shown by the MTT assay. Results are expressed as percentage of viability compared to the control (i.e., cells without toxicant). (B) Protein content of HepG2 cells co-exposed to ZnONPs supernatants and CuNPs. Values represent the mean $\pm$ standard deviation of at least three independent experiments. Asterisks denote statistically significant differences with respect to the effect produced by the CuNPs alone at each concentration tested (one-way RM-ANOVA followed by Dunnett's test); * $p < 0.05$, ** $p < 0.01$ and *** $p \leq 0.001$. ^a: The ZnONPs supernatants were obtained by centrifugation of ZnONPs suspensions.

and CuNPs (Fig. 4). DLS did not reveal the presence of any particle in these supernatants (Fig. S3), therefore zinc detected was present only in its ionic form (Table S1). The viability of HepG2 cells co-exposed to a mixture of ZnONPs supernatants and CuNPs was not reduced compared to that of cells exposed to CuNPs alone (Fig. 4A). On the contrary, an increase in cellular viability was observed, which led to significant ($p < 0.001$ to $p < 0.05$) differences for most of the supernatant co-exposure experiments. We also found that this effect was caused by addition of zinc sulfate as a source of zinc ion (Fig. 4A).

To determine whether the increase in viability observed in the MTT assay was related to an increase in the number of cells, optical microscopy observations were made and the protein content of the wells was measured. Exposure to ZnONPs supernatants (alone or together with CuNPs) caused a massive layer of hyper-confluent cells covering the bottom of the wells. At the same time, a substantial increase in the protein content of the wells was observed in cells treated with ZnONPs supernatants alone or when co-exposed to CuNPs (Fig. 4B).

3.4. Effects on HepG2 cell morphology and membrane

TEM was used to observe NP uptake and alterations of cell morphology or membrane integrity caused by individual NPs and mixtures. In the case of cells exposed to CuNPs alone at a concentration of 6.25 $\mu\text{g/mL}$, small particle clusters were observed dispersed in the

cytosol (Fig. 5A). Once the NPs had entered the cell, the presence of vesicles surrounding some of these clusters suggested that intracellular processing mechanisms were operating (Fig. 5B). The cells treated with ZnONPs alone also showed particle uptake, with NPs present in the cytoplasm and without evidence of membrane disruption (Fig. 5C). TEM micrographs of HepG2 cells co-exposed to CuNPs and ZnONPs with a diameter of 19 nm showed the cytoplasm full of membrane-bound vesicular processing bodies with a large number of particles inside (Fig. 5D). Regarding co-exposure to CuNPs and larger ZnONPs (57 nm), we detected vesicles containing small particles (Fig. 5E). These particles exhibited a morphology similar to that of the particles observed when cells were exposed to these CuNPs alone. This finding is shown in the inset at the bottom of image 5E. Also, large amounts of bigger particles resembling ZnONPs in their pristine form (top inset Fig. 5E) were present but not surrounded by any membrane (therefore we assume that they were not undergoing processing, Fig. 5E and F). EDX analysis allowed the positive identification of only copper (Fig. 5E). The detection of zinc was not possible, possibly due to the high background readings for osmium used during cell fixation. However, the morphology and size of some of the particles strongly suggest the presence of ZnONPs.

3.5. ICP-MS measurement

In order to obtain additional data to clarify the mechanisms behind the enhancement of toxicity observed in the co-exposure experiments, the total copper and zinc content of HepG2 cells after 48 h of exposure was determined by means of ICP-MS after the acid digestion.

The copper content of cells exposed to CuNPs alone increased from 1.4 to 1.6 μg copper/mg cellular protein when the nominal concentration of CuNPs in the exposure medium increased from 6.25 to 25.0 $\mu\text{g/mL}$ (Fig. 6). When cells were co-exposed to 6.25 $\mu\text{g/mL}$ CuNPs and to ZnONPs of different sizes, the cellular copper content ranged from 0.28 to 0.43 μg copper/mg cellular protein. The zinc content in the co-exposed cells increased with the increasing CuNPs concentration in the cell medium.

The zinc concentrations in the supernatants obtained after centrifugation of ZnONPs (6.25 $\mu\text{g/mL}$) in medium (Table S1) ranged from 0.18 to 0.25 $\mu\text{g/mL}$, which implies that the dissolution of ZnONPs in serum-free EMEM was less than 4% in this study.

4. Discussion

Previous studies have reported that the size, shape, and surface coatings of NPs are major factors affecting their bioavailability and toxicity. However, these NP features, in turn, are influenced by the composition of the media used in toxicity assays (George et al., 2012; Song et al., 2014). In this study, NPs showed a higher degree of aggregation than originally observed in the pristine preparations. This finding can be attributed to the culture media, EMEM, which contains high amounts of a variety of ions (1.8 mM of Ca^{2+} , 0.8 mM of Mg^{2+} , 5.4 mM of K^{+} , and 135 mM of Na^{+}) (Table S2) that favors such aggregation.

Here we found that the combination of two distinct NPs at concentrations causing no or very limited cytotoxic effects when present alone led to a strong enhancement of toxicity. Potentiation phenomena have been described for a variety of chemicals in a number of systems (for instance studies of Smith et al. (2013) in *D. magna* and Torre-Roche et al. (2013) in *C. pepo* and *G. max*). However, few data are available on such effects after exposure to a mixture of NPs (Chen et al., 2008; Tang et al., 2013).

In cells co-exposed to CuNPs and ZnONPs, the content of copper decreased with respect to that present after exposure to CuNPs alone, as determined by ICP-MS measurements. This finding suggests that copper is not responsible for the observed toxicity. At the same time, a considerable rise in the cell content of zinc was observed, accompanied by an increase in toxicity. Variations in copper and zinc cellular content could

Fig. 5. TEM images of HepG2 cells after 48-h exposure experiments. (A–B) HepG2 cells exposed to CuNPs alone (6.25 $\mu\text{g/mL}$). CuNPs were observed in the cytoplasm (A) and being processed by autophago/lysosomal machinery (white box) (B). (C) HepG2 cells exposed to ZnONPs (19 nm, 6.25 $\mu\text{g/mL}$) alone. The ZnONPs accumulated in the cytoplasm and appeared to be processed by endo/lysosomal machinery. (D–F) HepG2 cells co-exposed to CuNPs (6.25 $\mu\text{g/mL}$) and ZnONPs (19 nm, 6.25 $\mu\text{g/mL}$) (D) or (57 nm, 6.25 $\mu\text{g/mL}$) (E and F). Insets in (E) present EDX analysis and CuNPs (bottom) and ZnONPs (top) images extracted from micrographs of cells exposed only to CuNPs or to ZnONPs, respectively, and indicate that these NPs show a similar size and shape as those observed in the co-exposure experiments.

be attributable to NPs or to soluble ions. TEM analysis indicated the presence of NPs that were similar in shape to the pristine CuNPs or ZnONPs. In addition, EDX analysis corroborated the presence of CuNPs inside the cells. These approaches thus confirmed the internalization of both kinds of NP into the cell. Although we cannot draw a conclusion about the mechanism by which ZnONPs or CuNPs are taken into the cell on the basis of the TEM images obtained, we found no evidence supporting endocytosis. We recently reported that graphene nanoplatelets penetrate HepG2 and PLHC cells without any apparent

endocytosis (Lammel et al., 2013). Since the mechanism of cellular internalization of CuNPs and ZnONPs was not in the scope of this study, we performed no further studies in this direction; however, our results open up new avenues of research to elucidate this phenomenon.

To shed light on the mechanisms underlying the enhancement of toxicity observed after addition of ZnONPs and the increase in cellular zinc content, we attempted to differentiate the contribution of ZnONPs and of zinc ions to the elevated cytotoxicity. For this purpose, we

Fig. 6. Total zinc and copper content in HepG2 cells exposed to CuNPs alone or co-exposed to CuNPs and ZnONPs, as determined by ICP-MS.

performed co-exposure experiments using supernatants free of ZnONPs, as shown by DLS measurements, but containing zinc ions, as determined by ICP-MS analysis. Although the release of zinc ions has been proposed as the primary mechanism of ZnONPs toxicity in aquatic organisms (Franklin et al., 2007; Yoon et al., 2007), not only did co-exposure of HepG2 cells to ZnONPs supernatants and CuNPs not cause an increase in cytotoxicity, it led to greater cell viability. In previous studies using ZnONPs or CuNPs individually, we reported that cytotoxicity cannot be explained only by the ions released and that the direct interaction of NPs with cells is essential to induce the cytotoxicity of nanomaterials (Fernandez-Cruz et al., 2013; Song et al., 2014). The increase in cell viability after exposure to ZnONPs supernatants might be related to the nature of zinc as an essential element which, at the low concentrations (0.18–0.25 µg/mL) detected in the supernatants, exerts a positive effect on cell homeostasis and on the tolerance of HepG2 cells to CuNPs. A hormetic effect of zinc ions on the toxic impact of AgNPs on HepG2 cells and *Escherichia coli* has been reported (Kawata et al., 2009; Xiu et al., 2012). The presence of low amounts of zinc ions may activate cell repair mechanisms against these noxious substances, thus compensating the deleterious effects of the exposure.

It must be noted here that we were unable to establish a relationship between the zinc concentration of the supernatants and ZnONPs size. In a previous study, we found that the amount of copper ions released from CuNPs was not determined by NPs size (Song et al., 2014). This finding implies that, in addition to NP size, other factors contribute to the dissolution of these particles. Therefore, the toxicity caused by any nanomaterial is either directly or indirectly dependent on size.

The decrease in cellular content of copper after co-exposure to CuNPs and ZnONPs suggests that zinc competes with copper for cell uptake. It appears that this competition can lead to a preferential internalization of ZnONPs, leading to a decrease in the total content of copper inside the cell, which may also contribute to the detrimental effects observed. The mutual inhibition of the intestinal absorption by these two elements has already been reported (Hall et al., 1979; Hogstrand, 2011). Similarly, the inhibition of copper uptake has been reported in Caco-2 cells (Arredondo et al., 2006). In this cell line, an excessive intake of zinc results in overt copper deficiency, which has been exploited beneficially for the treatment of Wilson's disease to prevent copper accumulation (Barone et al., 1998).

In cells exposed to CuNPs alone, TEM analysis allowed observation of the presence of NPs surrounded by cellular membranes with a structure that could be assimilated to autophagosomes. In the co-exposure experiments, these kinds of membrane structures were seen only around NPs similar in shape and size to those inside the cells exposed solely to

CuNPs. However, these membrane structures were not found in cells exposed to ZnONPs alone, neither were they detected around NPs similar to ZnONPs in the micrographs of cells in the co-exposure experiments. These observations suggest that the CuNPs processing route is not affected by the presence of ZnONPs but rather that the opposite is occurring. Therefore, it is possible that zinc levels increase to reach a critical concentration that affects cell homeostasis, leading to cell death. As stated earlier, since supernatants containing only zinc ions did not cause toxicity when co-incubated with CuNPs, we deduce that the contribution of zinc to the toxicity was produced only by the nano-particulate fraction. TEM micrographs showed a number of relatively large ZnONPs aggregates or agglomerates inside the cells and close to the plasma membranes. These aggregates were not surrounded by membranes and therefore were not being processed by the cellular machinery. It is therefore plausible that these unprocessed ZnONPs damaged the cell membranes, thus causing cell death.

It has been widely considered that the toxicity of NPs is highly dependent on their size, with smaller NPs being more cytotoxic, which is generally attributed to an increase in their specific surface (Kim et al., 2012; Maurer-Jones et al., 2013; Unrine et al., 2010). However, the results of the co-exposure experiments performed in the present work differ from those obtained in studies using individual NPs. Here, addition of ZnONPs with the largest size (57 nm) to CuNPs caused the highest cytotoxicity in HepG2 cells. These findings are in agreement with the idea that the interaction of large ZnONPs aggregates with the membrane is one of the factors driving toxicity (Vakurov et al., 2013; Vandebriel and De Jong, 2012). Moreover, they indicate that the exposure of cells to combinations of NPs introduces new factors that greatly contribute to determining the global toxicity of the mixture.

5. Conclusion

Here we addressed the potentiation effect of ZnONPs at the non-toxic concentration and CuNPs on the cytotoxicity of HepG2 cells. We observed an enhancement of toxicity, thus pointing to a potentiation effect. We found that the enhancement of CuNPs toxicity produced by the addition of ZnONPs is attributable to the nano-particulate fraction (and not to zinc ions) and is influenced by the size of the ZnONPs. In addition, microscopy studies and the measurement of cellular concentrations of zinc and copper indicated that the build-up of large unprocessed ZnONPs aggregates or agglomerates might explain the increase in toxicity observed.

Although potentiation effects are considered when evaluating risk in specific cases, such as in workplace exposure or for pesticide residues in food and feed (Smith et al., 2013), most regulatory frameworks for chemicals continue to focus on the testing of single substances. However, here we demonstrate that the combined toxicity of several substances—a realistic scenario—can differ greatly from that produced by each individual substance alone.

Conflict of interest

No conflict of interest exists in the submission of this manuscript 'The potentiation effect makes the difference: Non-toxic concentrations of ZnO nanoparticles enhance Cu nanoparticle toxicity in vitro'.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <http://dx.doi.org/10.1016/j.scitotenv.2014.10.020>.

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