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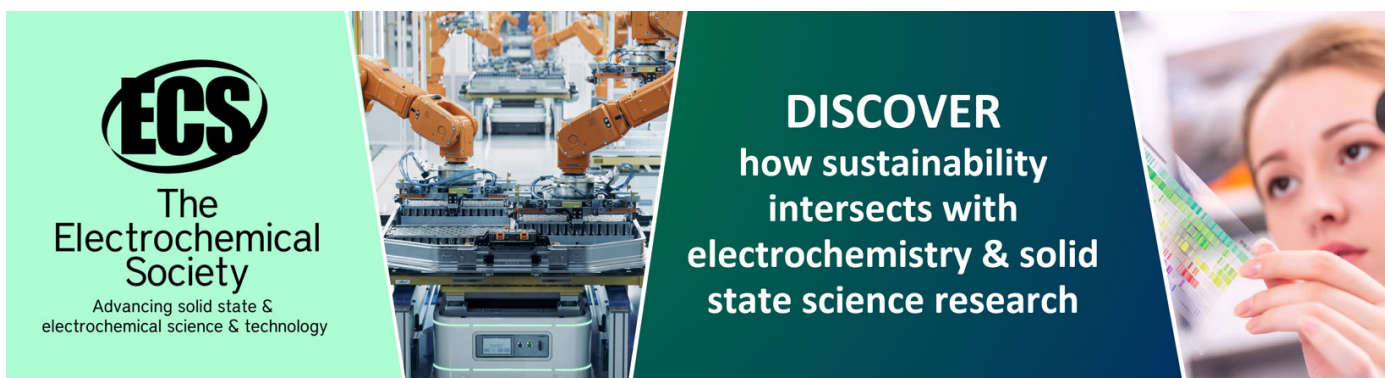
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Cytotoxicity effects of metal oxide nanoparticles in human tumor cell lines

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Abstract: Metallic and metal oxide nanoparticles (Nps) have a wide range of applications in various settings including household, cosmetics and chemical industries, as well as for coatings. Nevertheless, an in-depth study of the potential toxic effects of these Nps is still needed, in order to fulfill the mandatory requirement of ensuring the safety of workers, patients and the general public. In this study, Quick Cell colorimetric assays were used to evaluate the *in vitro* toxicity of different metal oxide Nps [Fe(II,III)O_x, TiO_x, ZnO and CeO₂] in several cell lines. The ZnO Nps were found to be highly toxic, with a lethal dose ≥ 100 $\mu\text{g/ml}$ for all the cell lines studied. Western blot was also used to test the ability of the different Nps to activate the complement pathway. However, no activation of this cascade was observed when the Nps were added. In addition, the aggregation state and charge of the Nps in culture media was studied by dynamic light scattering (DLS) and measurement of zeta potential. Transmission Electron Microscopy was used to analyze Np uptake and localization at the cellular level.

1. Introduction

The term nanoparticle (Np) applies to particles between 1 and 100 nm in at least two dimensions [1]. These particles have specific physicochemical properties that are not shown in the bulk form [2], most of the unique properties of Nps being defined by their high ratio of surface to volume, which implies that almost all the material is at the surface, and for the presence of quantum confining phenomena at the nanoscale. Nps offer, compared to bulk materials, a larger surface for adsorption, and sometimes a higher reactivity, interfering as catalyzators in many processes [3]. The huge potential of Nps for various applications makes the in-depth analysis of their potential toxicity in humans essential. In this context, a new discipline called Nanotoxicology has arisen, a branch of the toxicology and of the nanotechnology dealing with the interaction of nanomaterials, nanostructures and devices with biological molecules and organisms, aiming to understand possible toxicological side-effects of the

Nps [2]. One of the scopes of nanotoxicology is the evaluation of the safety of Nps for industrial applications, for providing information about the undesirable effects of the Nps, and for developing the tools to prevent such effects.

Two main mechanisms might be responsible for an eventual toxicity of metallic and metal oxide Nps. First, the intrinsic catalytic activity of such Nps can disturb several processes and intracellular signaling pathways. Second, ions can also be released from the Np, affecting the finely regulated concentration of metallic ions inside the cell. The Nps could induce toxicity in several organs, even leading to systemic toxicity [1]. They could also affect the immune system, activating or inhibiting it, by inducing allergic responses or hypersensitivity, the generation of antibodies against the Nps or their coatings, or by activating cells from the endothelial reticulum system [4].

In this study, using different Np concentrations in several cell lines, *in vitro* cytotoxicity analyses and endocytosis assays were undertaken to evaluate the potential toxicity of metal oxide Nps.

2. Materials and methods

2.1. Nps

The Fe(II,III)O_x and TiO_x Nps were supplied by PlasmaChem (Berlin, Germany), and the ZnO and CeO₂ Nps by Evonik Degussa (GmbH, Germany) (Fig.1). The concentration of Fe(II,III)O_x Nps was 72 mg/ml, and for the remaining Nps, stock solutions of 72 mg/ml in PBS were prepared. The sterility of the Nps was preserved in all cases.

2.2. Cells

The toxicity assays were performed in the human tumoral cell lines A549, NCI-H460, SK-MES-1 and HeLa, all purchased from ATCC (American Type Culture Collection). All cell types were cultured in RPMI medium (GIBCO Invitrogen Corp., Grand Island, NY), supplemented with 10% FBS, at 37°C, 5% CO₂.

2.3. Aggregation studies

For evaluating the behavior of the Nps in the physiological medium RPMI with 10% FBS, a 24-well plate was used, in which aggregation was assessed for the sonicated or unsonicated Nps. The 20 min sonication was carried out in a Branson ultrasound bath (Branson 1510, Danbury, CT), at low frequency (47 kHz), preventing Np exposure to potential contaminating agents. For these assays, the final concentration of the Nps was 8 µg/ml, in 1 ml final volume. The Nps were incubated overnight at 37°C and the formation of aggregates was evaluated by optical microscopy in an inverted microscopy model IX50 from Olympus with 20× and 40× objectives (Olympus Optical Co, GMBH, Germany).

2.4. Dynamic light scattering (DLS)

A Malvern NanoZS device(UK) was used for DLS measurements.

2.5. Cellular proliferation colorimetric assay

To measure the effect of the Nps on cellular viability, the colorimetric kit Quick Cell Proliferation Testing Solution (GenScript Corporation, Piscataway, NJ, USA) was used following the instructions of the manufacturer. Once the optimal cell number for each cell line was determined, the viability assay was performed including the Nps at three different concentrations, 0.5 µg/ml, 50 µg/ml and 1 mg/ml, respectively. Briefly, cells were incubated with 200µl RPMI 10% FBS, both in the absence and in the presence of Nps, for 24 and 48 h. The plates were then centrifuged at 1000 g for 1 min, 100 µl of the supernatant were discarded and 50 µl of Quick Cell reagent added. Subsequently, the plates were incubated for 4h, centrifuged again and the supernatants were transferred to clean plates, to avoid possible interference due to the Nps. Finally, the absorbance was measured at 450 nm in an Envision multidetector (Perkin Elmer Inc., Norwalk, Connecticut, USA). As a positive toxicity control, cells were incubated with 5% Triton X-100 (Sigma-Aldrich, Steinheim, Germany), which was

removed 2 hours before 24/48 h; in wells used as a control of cellular death, 100 μ l of medium was added. As a negative control, RPMI and Nps alone were used. The results were then analyzed by the following equation:

$$\% \text{ cellular viability} = \frac{\text{Abs Cells} + \text{Nps} - \text{Abs Nps}}{\text{Abs Cells} - \text{Abs RPMI}} \times 100$$

For measuring the lethal dose of the ZnO Nps, the same assay was performed, incubating the cells with intermediate concentrations of these Nps, ranging from 0.5 up to 500 μ g/ml.

2.6. Analysis of apoptosis and necrosis

The FITC Annexin V Apoptosis Detection Kit I from BD Biosciences (San Diego, USA) was used according to the manufacturer's instructions. Briefly, cells cultivated in exposure media were stained with Annexin V-FITC, which binds to phosphatidylserine at the outer cell membrane and can, therefore, be used as a marker to detect apoptosis. Propidium iodide (PI) was used to quantify necrotic cells. The dye stains DNA after the membrane becomes permeable for PI. Stained cells were then analyzed by flow cytometry (FACSCalibur, Becton Dickinson, Mountain View, USA) at an excitation wavelength of 488 nm. FITC and PI fluorescence were detected in the green and red fluorescence channels, respectively.

2.7. Complement activation

Western blot with an anti-C3 antibody was carried out to analyze the degree of degradation of this factor upon Np addition. A pool of human sera from healthy donors was incubated with two different concentrations of Fe(II,III)O_x, TiO_x, CeO₂ and ZnO Nps (0.5 and 50 μ g/ml). Cobra venom (CVF, Quidel Corporation, San Diego, CA, USA), and PBS were used as positive and negative controls, respectively. The membrane was revealed with an antibody specific for C3b from Abcam (Cambridge, UK).

2.8. Scanning electron microscopy

Cells were prefixed in 2.5% glutaraldehyde/0.1 mol/L sodium cacodylate (pH 7.4). After postfixation in 1% osmium tetroxide (in 0.2 mol/l cacodylate buffer), cells were dehydrated in a series of increasing ethanol concentrations and at the critical point dried using carbon dioxide. After coating with gold, cells were examined with a JEOL JSM-6700F scanning electron microscope.

3. Results and discussion

Metal oxide Nps are widely employed in creams, implants, drug carriers or as contrast agents [5], but for the potential use of these Nps in biomedicine strict toxicity rules should be followed. The Nps should also be stable in physiological conditions, not forming aggregates that could obstruct the capillaries. To test aggregation, we immersed the Nps in physiological medium and found that all Nps were aggregated in these conditions (not shown), with the smallest aggregates corresponding to the ZnO Nps. To disperse the NPs these were sonicated, but this proved to be only partially successful (Figure 1a). To further address the aggregation status of the Nps, dynamic light scattering (DLS) experiments were carried out, dispersing the particles in different media, with some Nps being sonicated, while others were left unsonicated. Concurring with the results of previous studies, a strong aggregation effect was observed in all the Nps analyzed (Figure 1b and not shown). This clearly represents an important limitation for the *in vivo* use of these Nps.

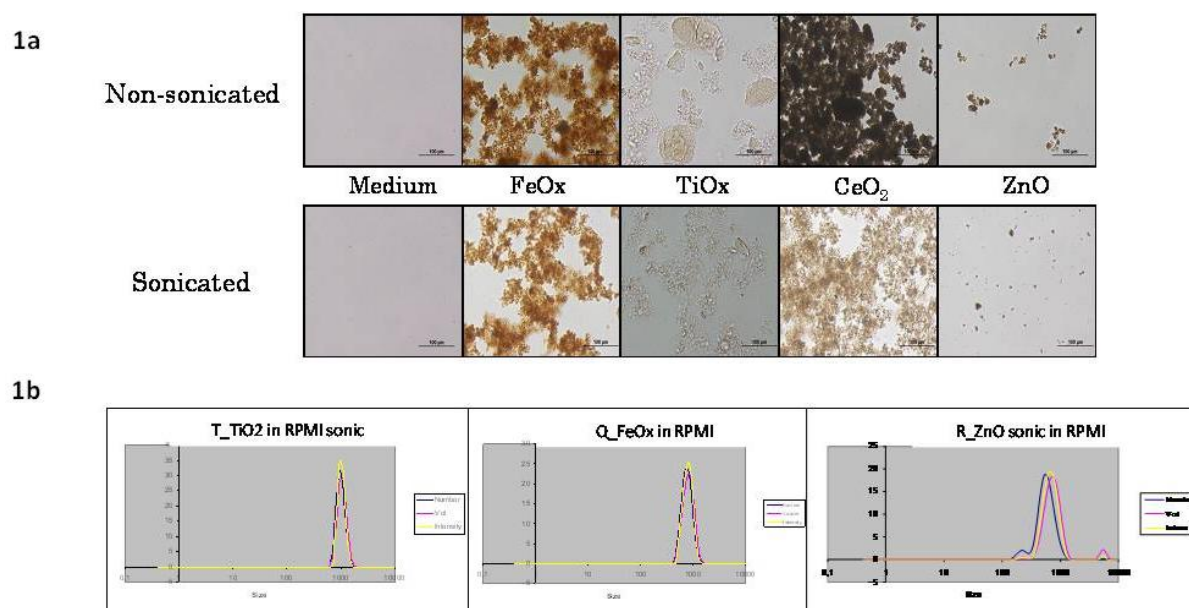


Figure 1. Aggregation of the Nps. (1a) Optical microscopy images depicting the aggregation of the Nps before and after sonication. The Nps were used at 8- μ g/ml concentration and incubated overnight. Bar: 100 μ m. (1b) Aggregation status of TiO_x, FeO_x and ZnO Nps measured by DLS. All the Nps show a tendency to form aggregates in RPMI medium.

The use of *in vitro* assays of cellular viability in different cell lines is essential for evaluating the potential toxicity of the Nps. In this study, we employed toxicity studies based on colorimetric methods. For an Np to be considered non-toxic, we followed the criteria of the Nanotechnology Characterization Laboratory (NCL) in Frederick (Maryland, USA), especially that cell viability at 48 h should be higher than 75%. Using the Quick Cell assay, it was seen that ZnO Nps induced a massive toxicity in all the cell lines studied at 24 and 48 hours (Figures 2 a-d), in agreement with previous studies by other research groups [6-8]. This could be due to either the presence of small aggregates formed by ZnO Nps, which could have greater interaction with the cell causing its death, or to the release of Zn⁺² ions from the Nps, which are also toxic.

These assays were optimized, as cellular metabolism varies significantly among cell lines and viability can be affected by factors, such as cell density, percentage of living cells with respect to dead ones, and different proliferation rates. Once the optimal amount of cells to be analyzed for all the cell lines studied was determined (data not shown), we were able to carry out dose-response viability assays with these Nps, and found that the lethal dose 50 (LD 50), able to cause the mortality of at least 50% of the cells, was ≥ 100 mg/ml for all the cell types studied (Figures 3a-d).

In addition, the population of necrotic and apoptotic A549 cells exposed to ZnO Nps, and stained with annexin V-FITC/PI was analyzed by flow cytometry. The percentage of viable and dead cells (apoptotic, necrotic and late apoptotic cells) as a function of exposure time and concentration are shown in Figure 3e. With higher Np concentrations and longer exposure times the number of dead cells increased, mainly late apoptotic and apoptotic ones, with the latter only seen after 24-h exposure time. Although 3 μ g/ml ZnO Nps only had a small impact on A549 cells, 30 and 100 μ g/ml ZnO Nps considerably reduced cell viability. Even after 12-h exposure at Np concentrations of 30 and 100 μ g/ml, treated cells did not match the "Acceptance Criteria". Yet, ZnO concentrations of 3 μ g/ml are considered non-toxic for A549 cells, even after 72-h treatment with the Nps.

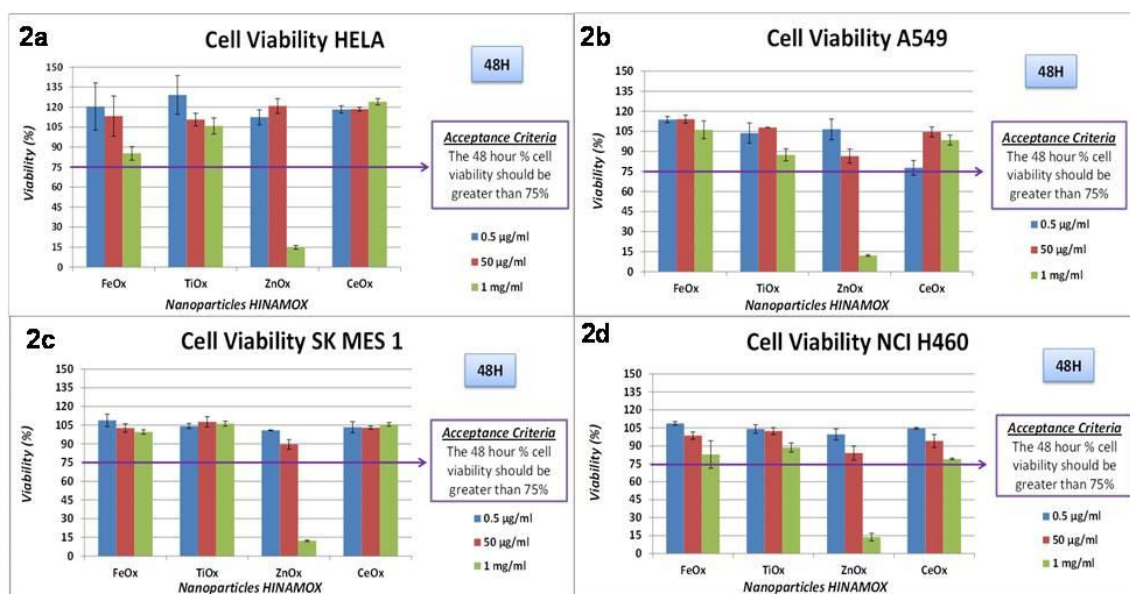


Figure 2. Effect of the different Nps on cell viability. Graphs showing the effect on cell viability of the different Nps, by Quick cell colorimetric assay, at 48 h, in different cell types: HeLa (2a), A549 (2b), SK-MES-1 (2c) and NCI-H460 (2d).

Nps were considered non-toxic if at 48h cell viability reached at least 75%. All the assays were performed twice for each cell line, at 24 and 48 h, and in triplicate for each Np concentration.

Another important limitation to be taken into account regarding the potential *in vivo* use of Nps is that they are quickly removed from the circulation by the endothelial reticulum system, which would make the use of these compounds as drug carriers or contrast agents more difficult. Nevertheless, covering the Nps with agents such as polyethylenglycol can improve their permanence in the circulation, thereby avoiding recognition by the cells of the endothelial reticulum system [9]. In our study, transmission electronic microscopy was used to evaluate the intracellular localization of TiO_x Nps (Figure 4). After 6-h incubation, a large amount of particles adhered to the cell membrane, and the cells appeared to have ingested some of these particles (Figure 4c). Most of the particles seemed to be confined inside vesicles distributed across the cytoplasm, not crossing into the nucleus. At 24-h incubation time with the Nps, particles were also seen to be attached to the cell membrane, suggesting that the cells had ingested a relatively large amount of these compounds. The vesicles moved towards the nuclear membrane, but few had penetrated inside the nucleus (Figure 4d).

Possible activation of the complement, a key component of the innate immune system consisting of a cascade of more than 30 factors, should also be taken into consideration before *in vivo* use of Nps. It is well established that, *in vitro*, many types of particle are able to activate the alternative pathway of the complement [10]. In our study, western blot was used to evaluate the ability of the Fe(II,III)O_x , ZnO, CeO_2 and TiO_x Nps to activate the complement. The use of a specific antibody against the C3 factor makes it possible to evaluate the degradation of this protein, which corresponds to activation of the complement. It was seen that none of the Nps assessed was able to induce complement activation (Figure 5), as the C3 factor remained largely intact, with basal degradation similar to that of the negative control.

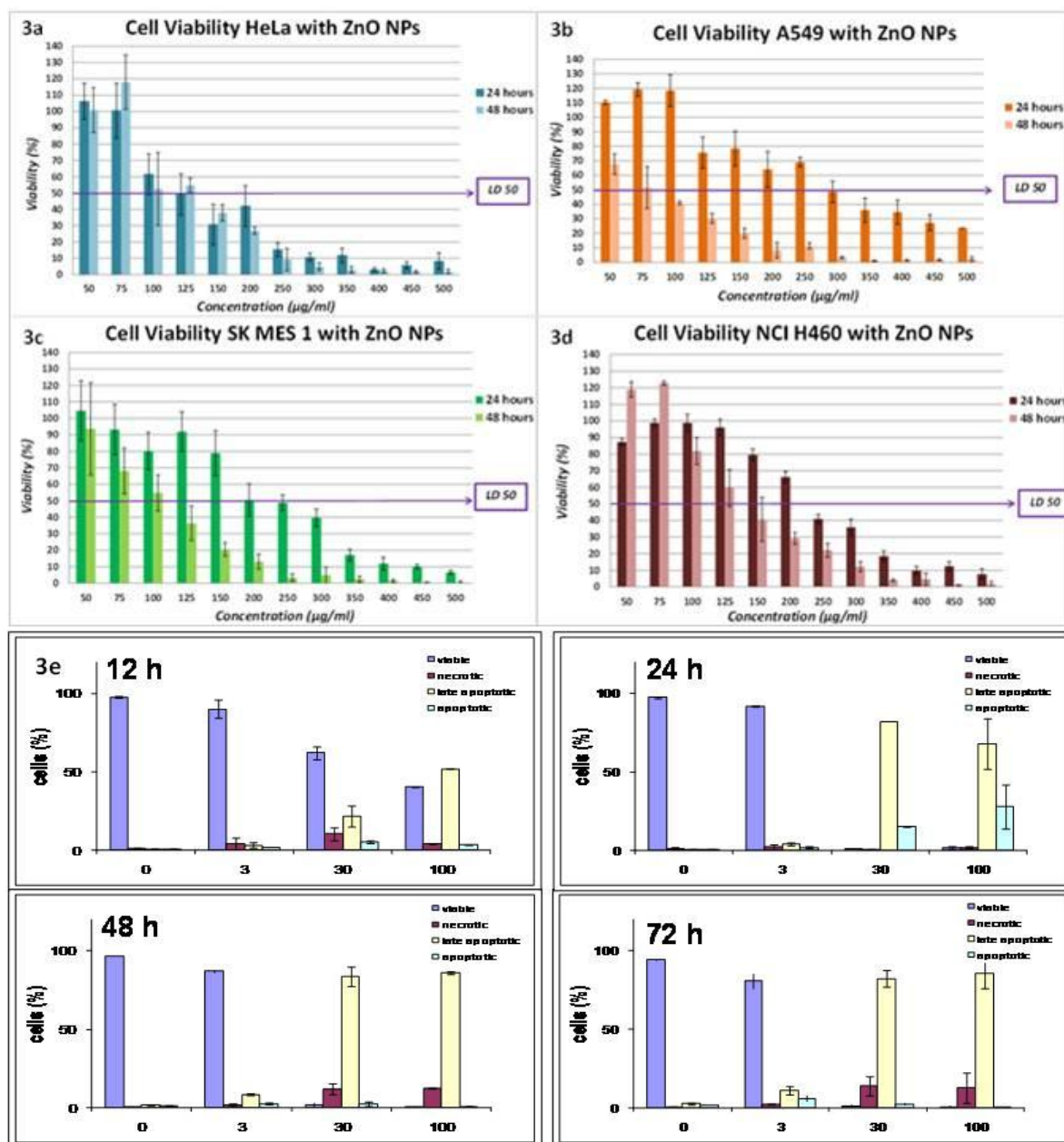


Figure 3. Determination of the lethal dose 50 (LD 50) for ZnO Nps in the different cell lines. Effect on cell apoptosis and necrosis of ZnO Nps: HeLa (3a), A549 (3b), SK-MES-1 (3c) and NCI- H460 (3d) by Quick cell colorimetric assay, at 24 and 48 h, using increasing concentrations of these Nps, as indicated. The LD 50 was reached at ZnO Np concentrations $\geq 100 \mu\text{g/ml}$ for all the cell types. (3e) Impact of ZnO nanoparticles on the viability of A549 cells stained with annexin V-FITC/PI and measured by flow cytometry. Cells were incubated with the indicated Np concentrations, ranging from 3 to $100 \mu\text{g/ml}$, at different time points (12, 24, 48 and 72 h). Fractions of viable, apoptotic, late apoptotic and necrotic cells (%) were determined.

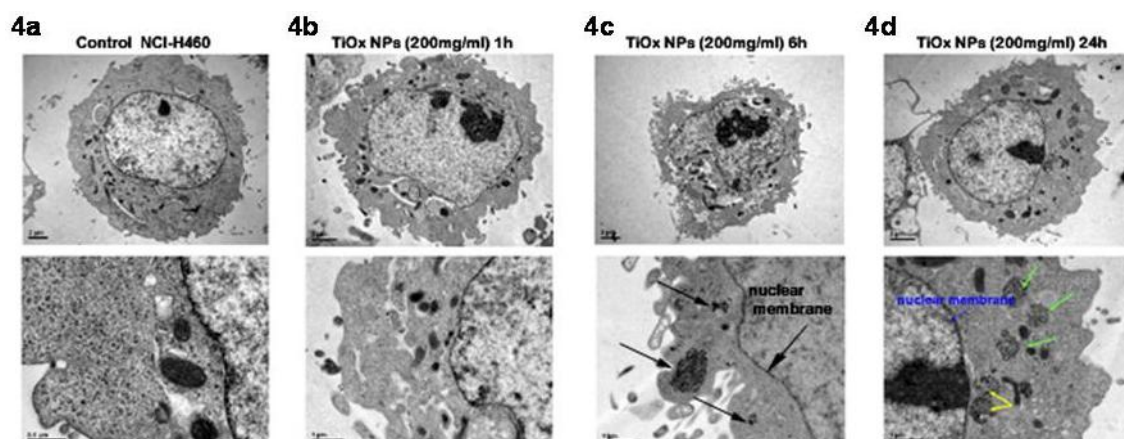


Figure 4. Subcellular distribution of TiO_x Nps in NCI-H460 cells. Transmission electron microscopy (TEM) images of microtomed sections of HCI-H460 cells untreated (4a) or incubated with 200 $\mu\text{g}/\text{ml}$ TiO_x nanoparticles at different time points, from 1 up to 24 hours (4b-d). The small black dot and the large black regions correspond respectively to glycogen and mitochondria. Almost no particles were found around or inside the cells after 1-h Np incubation (4b). After 6-h incubation, a large amount of particles adhered to the cell membrane (left), and the cells seem to have ingested some particles (4c). At higher magnification, many particles can be seen attached to the membrane, observing clusters with the indented cell membrane in the process of endocytosis (top), and endosomes filled with particles. Most of the Nps seem to be confined inside the vesicles distributed across the cytoplasm, not crossing into the nucleus (4c). At 24-h incubation time, particles are also observed attached to the cell membrane, suggesting that the cells have ingested a relatively large amount of particles, and two of the vesicles close to the nucleus seem to be in the process of fusing together. The vesicles moved towards the nuclear membrane, but few have penetrated inside the nucleus (4d).

Bars: 2, 1 and 0.5 μm .

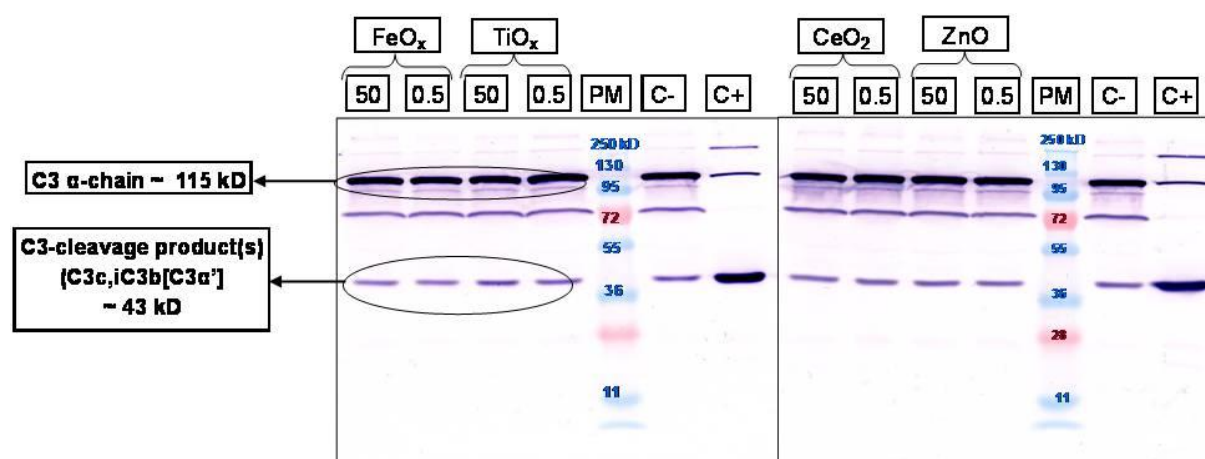


Figure 5. The complement system is not activated by the Nps. The degradation of the complement factor C3 was analyzed by Western blot and revealed with an anti-C3 antibody. A pool of sera from different healthy donors was incubated with the Nps indicated, at 0.5 and 50 $\mu\text{g}/\text{ml}$, with cobra venom as a positive control of C3 degradation or with PBS as negative control. The bands of 115 kD and 43 kD correspond to the intact C3 factor and main degradation products of this protein, respectively.

4. Conclusion

In summary, taking into account the information available and the data obtained in this study, the Nps analyzed show a tendency to form aggregates in media containing serum. In addition, the ZnO Nps were found to be toxic, with a lethal dose ≥ 100 $\mu\text{g/ml}$ for all the cell lines studied. The amount of dead cells increased with rising Np concentration and exposure time, but an increase of apoptotic cells was seen only after 24h. Moreover, most of the Nps seemed to be confined inside the vesicles distributed across the cytoplasm, not crossing into the nucleus and none of the Nps assessed were able to induce complement activation.

Further in-depth research of these aspects is, therefore, still required to determine the potential toxic effects on human health.

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References

- [1] Hussain, S.M., Toxicity Evaluation for Safe Use of Nanomaterials:Recent Achievements and Technical Challenges. *Advanced Materials*, 2009. **21**: p. 1549-1559.
- [2] Arruebo, M., M. Valladares, and Á. González-Fernández, Antibody-Conjugated Nanoparticles for Biomedical Applications. *Journal of Nanomaterials*, 2009.
- [3] Borm, P.J. and W. Kreyling, Toxicological hazards of inhaled nanoparticles--potential implications for drug delivery. *J Nanosci Nanotechnol*, 2004. **4**: p. 521-31.
- [4] Hu, X., et al., In vitro evaluation of cytotoxicity of engineered metal oxide nanoparticles. *Sci Total Environ*, 2009. **407**(8): p. 3070-2.
- [5] Moghimi, S.M., A.C. Hunter, and J.C. Murray, Nanomedicine: current status and future prospects. *FASEB J*, 2005. **19**(3): p. 311-30.
- [6] Heng, B.C., et al., Toxicity of zinc oxide (ZnO) nanoparticles on human bronchial epithelial cells (BEAS-2B) is accentuated by oxidative stress. *Food Chem Toxicol*, 2010. **48**(6): p. 1762-6.
- [7] Moos, P.J., et al., ZnO particulate matter requires cell contact for toxicity in human colon cancer cells. *Chem Res Toxicol*, 2010. **23**(4): p. 733-9.
- [8] De Berardis, B., et al., Exposure to ZnO nanoparticles induces oxidative stress and cytotoxicity in human colon carcinoma cells. *Toxicol Appl Pharmacol.*, 2010. In press.
- [9] Zahr, A.S., C.A. Davis, and M.V. Pishko, Macrophage uptake of core-shell nanoparticles surface modified with poly(ethylene glycol). *Langmuir*, 2006. **22**(19): p. 8178-85.
- [10] Wilson, M.R., Gaumer, H.R., Salvaggio, J.E., Activation of the alternative complement pathway and generation of chemotactic factors by asbestos. *Journal of Allergy and Clinical Immunology*, 1977. **60**(4): p. 218-222.