How does the deposited dose of oxide nanomaterials evolve in an in vitro assay?

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Abstract. In this work, the evolution of some key physicochemical parameters of oxide engineered nanomaterial (ENM) dispersions was studied during an in vitro biological assessment. Commercial oxide ENMs, SiO2 and TiO2, were dispersed in aqueous solutions (20 µg/mL) to A549 cells and N-hTERT keratinocytes and were assessed at several incubation times: 6, 24, 48, and 72 hours. The ENMs deposited dose and its particle size distribution (PSD) were followed each time. Centrifuge Liquid Sedimentation (CLS) measured the PSD and the ENMs deposited dose from a particle entitiy perspective, while Particle-Induced X-ray Emission (PIXE) measured the ENMs deposited dose from an elemental mass perspective. No significant variations in PSD were observed for SiO2 ENMs during incubation in A549 cells and TiO2 ENMs in both cell lines, while a continuous evolution of the PSD is observed for SiO2 in N-hTERT keratinocytes. The deposited dose for TiO2 ENMs remained stable and similar in both cell lines due to a smaller specific surface area and a higher quantity of primary particles present during incubation. It is concluded that the observed differences in the deposited dose are related to an interaction between the proteins present in the media and the ENMs specific surface.

1. Introduction
Engineered nanomaterials (ENMs) exhibit remarkable properties, which are fundamentally different to those of bulk materials. These properties induced a rush in industry to produce and use these new materials in different products, touching wide and diverse areas such as semiconductors [1], medicine [2], cosmetics [3], and food [4, 5]. However, stakeholders have raised health concerns due to the unprecedented progress uncoupled with a proper understanding of the possible associated risks. Indeed, several reports have indicated that ENMs may be more toxic than their larger counterparts [6-9].
The field of nanotoxicology, and more broadly nanosafety, was then established with the aim to assess the potential hazard of nanomaterials [10]. Among the different efforts in the field, the *in vitro* approach allows to investigate the intracellular pathways induced by ENMs [8] and their consequences [11-13]. A relatively recent acknowledgement is the need of ENMs physicochemical characterization [11, 14, 15], not only as dry powders or aqueous predispersions but also as suspensions in cell culture medium added to their “behaviour” evaluation during *in vitro* exposure. Commercial ENMs are not always only composed of primary particles (PPs) but can also contain agglomerates and/or aggregates [10]. Once dispersed in liquid milieu the PPs/agglomerates-aggregates ratio could evolve and be influenced by sedimentation (sub-micron particles) and diffusion (nanometer-sized particles) [16]. In this respect there have been efforts to understand ENMs dynamics in cell culture media, both experimentally [17] and computationally [18]. Even if the general principles are understood in cell culture media, other parameters should play a role during cell lines incubation, such as the cell culture media consumption as a function of incubation time and cell growth. There is not any generic information about the ENMs deposited dose during a specific cell line incubation nor a description of the key parameters that play a role in predicting this deposited dose.

Oxides are a class of ENMs that are currently used for several commercial applications. For example, silica (SiO$_2$) is used as a food additive [4], and titania (TiO$_2$) is used in cosmetic products such as sunscreens [3]. These ENMs, which are part of the OECD ENM list [19], were chosen as relevant model oxides due to their current uses. Human alveolar epithelial A549 and Human telomerase-immortalized (N-hTERT) keratinocytes [20] were chosen to represent two of the most important exposure routes to ENMs [21].

The aim of this work is to study the evolution, with two complementary techniques for PSD and weight determination, of the SiO$_2$ and TiO$_2$ ENMs deposited dose at 20 µg/mL after increasing incubation times (6, 24, 48, and 72 hours), on A549 cell lines and N-hTERT keratinocytes.

2. Materials and methods

2.1. ENMs

SiO$_2$ ENMs dry powders were supplied by Nanologica™ through the NanoValid project (FP7 2007-2013). This is a mesoporous ENM synthesized via sol-gel. TiO$_2$ ENMs dry powders were purchased from Sigma Aldrich (CAS number 13463-67-7). Both ENMs were employed as received.

2.2. ENM stock dispersions

A stock dispersion of each ENM was prepared at a 1 mg/mL (w/v) concentration in ultrapure water and magnetically stirred during 15 min at 400 rpm. A 48 µL volume of the stock solution was added to each cell culture box containing each of the two culture media, specific to each cell line, to achieve a final ENM 20 µg/mL concentration.

2.3. Cell lines

Human alveolar epithelial A549 cells were purchased from the American Type Culture Collection (ATCC). A549 cells were cultured in minimum essential medium (MEM, Gibco) plus serum. MEM contains 10% fetal bovine serum (Gibco) and 1% penicillin–streptomycin (BioWhittaker). This culture medium will be referred throughout this paper either as MEM plus serum or A549 culture medium.

Human telomerase-immortalized keratinocytes (N-hTERT) [20] were kind gifts from Dr. J.G. Rheinwald (Department of Medicine and Harvard Skin Disease Research Center, Boston, MA, USA). They were maintained in Epilife medium with human keratinocyte growth supplement (Cascade Biologics) and antibiotics (penicillin/streptomycin, BioWhittaker).

Both cells were grown in a 5% CO$_2$ incubator in a humidified 37°C atmosphere.

2.4. In vitro assay
995,000 A549 cells or 900,000 N-hTERT keratinocytes were seeded in 25 cm$^2$ polystyrene flasks (T25, Corning) and incubated with 20 µg/mL (6 mL of culture medium/T25 was used) of either SiO$_2$ or TiO$_2$ ENMs for 6, 24, 48 and 72 hours (one flask was incubated for each incubation time). After incubation, the cell culture medium was collected by tilting the cell boxes towards one of the bottom corners and then stored in glass containers.

2.5. ENMs characterization

2.5.1. Dry powders. The average pristine ENMs diameter was obtained with a Field Emission Gun Scanning Electron Microscope (FEG-SEM) Jeol JSM 7500 operated at 20 keV. Both ENMs bulk composition was measured with an EDX (Energy Dispersive X-ray) detector coupled to the FEG-SEM. Each powder was deposited on a double face carbon tape.

Specific Surface Area (SSA) measurements were obtained by the BET method with an Accelerated Surface Area and Porosimetry System (ASAP, Micromeritics 2010).

Surface composition was analysed with an X-ray Photoelectron Spectroscopy (XPS) system. The used apparatus is a K-Alpha (Thermo Scientific) system using Al K-α X-rays, with spectra recorded at 90° take-off angle. The XPS analysis depth is around 5 nm. Core-level lines (C1s, Si2p) were calibrated to the C1s peak (284.6 eV). The spectra were analysed, fitting the Gaussian function to the experimental curve, with a non-linear least squares scheme, and using a Shirley background. Nominal resolution was measured as full width at half maximum of 1.0 eV (core-level spectrum) to 1.5 eV (survey spectrum). Each powder was deposited on a double face carbon tape.

2.5.2. ENM characterization during in vitro incubation. After incubation, the culture medium was collected and two 0.5 mL aliquots were taken: one for Centrifuge Liquid Sedimentations (CLS) and one for Particle-Induced X-ray Emission (PIXE) measurements. They were analysed within an hour in the case of CLS. Three independent experiments were conducted and the average and standard deviations were calculated.

Particle size distributions (PSD) (in number and weight) were measured with a DC24000 system Disc Centrifuge (CPS Instruments Inc.). This measurement is based on the CLS method according to Stokes’ law using a 405 nm wavelength laser. This method is also known as Differential Centrifugal Sedimentation (DCS). The diameters measured are hydrodynamic diameters, which provide the real diameter (sphericity factor equal to 1) for spherical particles. A certified PVC microparticle calibration standard (226 nm), provided by the instrument supplier, was used to calibrate all measurements. Each measurement was done by injecting a 0.5 mL volume.

PIXE was used for ENM quantification in cell medium. The principles of PIXE have been previously reported [22, 23]. For this study, a proton ion beam with a 2.5 MeV energy was used at low currents (< 1 nA) in order to avoid changes due to heating in the matrix. Samples were prepared in the following way: a truncated conical shape sample holder was used, with the following dimensions: 2 cm in diameter, 4 mm of depth and 2 mm in the truncation. This holder was designed as to hold a droplet for drying and preventing horizontal spread. Double face carbon tape was placed on top of the holder, providing a conductive surface and almost no background noise for the PIXE measurements. A certified PVC microparticle calibration standard (226 nm), provided by the instrument supplier, was used to calibrate all measurements. Each measurement was done by injecting a 0.5 mL volume.

The deposited dose is defined as the ENM dose that reaches the cells by either sedimentation (sub-micron particles) or diffusion (nanometer-sized particles). It was calculated, independently with CLS and PIXE techniques, from ENM content measurements in the cell culture medium. For CLS, the estimation was the difference between the integrated particle weight distributions at each incubation time compared to the control. For PIXE, the estimation was the difference between the measured ENM elemental concentrations at each incubation time with respect to a control with the applied dose (20 µg/mL).
3. Results and discussion

3.1. ENMs characterization (dry powders)

The average diameter of both dry ENMs, calculated from SEM images, was 197.5 ± 40.5 and 25.6 ± 4.9 nm for SiO$_2$ and TiO$_2$, respectively. In the case of SiO$_2$ ENMs, the average diameter was calculated from the particles with more than 100 nm, and not from the small particles observed at the bottom (cf. CLS results in section 3.2.1). A sample SEM image of each ENM is presented in Figure 1.

![SEM micrographs of a) SiO$_2$ NPs and b) TiO$_2$ NPs.](image)

**Figure 1.** SEM micrographs of a) SiO$_2$ NPs and b) TiO$_2$ NPs.

The XPS surface composition spectra for both ENMs are presented in Figure 2, and summarized along with other ENMs physicochemical properties in Table 1. It is noticeable that the SiO$_2$ SSA was more than one order of magnitude greater than that of TiO$_2$. In terms of composition, both ENMs at the surface are close to their stoichiometric 1:2 metal ratio (Si or Ti) to O, while the C content values are assumed to be due to environmental and synthesis contaminations. At bulk composition, SiO$_2$ contains more Si than O, a 1.5:1 ratio Si to O, as well as a considerable amount of C (26.3 %). The TiO$_2$ bulk composition kept a 1:2 stoichiometric ratio, highlighting that the C content is mostly located on the surface.

![XPS surface composition spectra of a) SiO$_2$ NPs and b) TiO$_2$ NPs.](image)

**Figure 2.** XPS surface composition spectra of a) SiO$_2$ NPs and b) TiO$_2$ NPs. The constituent elements atom percentage is presented.
Table 1. Physicochemical characterization of pristine SiO$_2$ and TiO$_2$ ENMs.

| Physicochemical property           | SiO$_2$       | TiO$_2$       |
|-----------------------------------|---------------|---------------|
| Average diameter (SEM) nm         | 197.5 ± 40.5  | 25.6 ± 4.9    |
| Specific surface area m$^2$/g      | 962           | 32            |
| at. % Si       | 43.7          | 27.4          |
| O       | 30.0          | 65.3          |
| C       | 26.3          | 7.36          |
| Surface composition (XPS)         | 32.6          | 27.4          |
| at. % Si       | 32.6          | 22.9          |
| C       | 63.7          | 55.9          |
| O       | 3.7-22.9      | 21.2          |

* Product purity reported as TiO$_2$ only.

3.2. ENMs size distribution

3.2.1. Size distribution in ENMs stock dispersions. The stock ENMs dispersions PSD is presented in Figure 3. The PSD was evaluated in terms of weight or volume distribution (emphasis on agglomerates/aggregates) and in number distribution (emphasis on PPs). Both ENMs weight distribution, Figure 3a and 3c, shows the presence of agglomerates/aggregates and sub-micron particles. In the case of SiO$_2$ the particles around 150 nm correspond to the agglomerates observed by SEM, see Figure 3a. For TiO$_2$ the weight distribution shows an even distribution between PPs and agglomerates/aggregates, see Figure 3c. The PPs for SiO$_2$ have an average hydrodynamic diameter of 20 nm and 18 nm for TiO$_2$, according to the number distributions in Figures 3b and 3d, respectively. Both of these ENMs, even though containing significant agglomerates/aggregates when dispersed, are classified as nanomaterials in accordance to the recommendation of the European Commission for the definition of a nanomaterial: more than 50% of particles in number being less than 100 nm in one of their dimensions [24].

Figure 3. CLS PSD of the ENMs in the stock dispersion: a) SiO$_2$ weight related distribution, b) SiO$_2$ number related distribution, c) TiO$_2$ weight related distribution, d) TiO$_2$ number related distribution. The percentages of particles smaller and bigger than 100 nm are indicated in the number related distributions.
3.2.2. Size distribution on cell culture medium. The PSD of SiO$_2$ and TiO$_2$ ENMs in MEM plus serum and Epilife cell culture media at several incubation times are presented in Figures 4 and 5. The cut-off size for PPs was 60 nm SiO$_2$ and 40 nm TiO$_2$, values that were determined from the CLS number related distribution (see Figures 3b and 3d, respectively). In the A549 culture medium, SiO$_2$ ENMs PSD stabilized with time. Such a behaviour is associated to the progressive filling of the open pores on the surface and to the presence of proteins in the medium, helping in the stabilization and de-agglomeration processes [25]. The TiO$_2$ ENMs PSD in the A549 culture medium was nearly stable at all incubation times.

In Epilife cell medium, the SiO$_2$ ENMs PSD presents a de-agglomeration with time. Such a de-agglomeration is associated to the culture medium type and to SiO$_2$ ENMs physicochemical properties. The SiO$_2$ ENMs high SSA and porosity and the higher content of Si in the core are signatures of a core-shell structure. Given that Epilife is a protein free medium (no serum), this medium likely fills the SiO$_2$ ENMs open pores, inducing a de-agglomeration process. In the case of TiO$_2$ ENMs in Epilife, its PSD remains unchanged with time. This result is due to the TiO$_2$ ENMs hard core structure, which is not strongly affected by the de-agglomeration process.

![Graph](image)

**Figure 4.** Percentage of PPs (---) and agglomerates (— —) from CLS measurements of a) SiO$_2$ ENMs and b) TiO$_2$ ENMs dispersed in MEM plus serum culture medium at a 20 µg/mL nominal dose during increasing incubation times with A549 cells. Values for incubation time of “0 h” correspond to the water pre-dispersion before contact with the culture medium. Both dashed lines are guides to the eye.

3.3. Deposited dose
The deposited dose (see last paragraph of Materials and Methods section) of SiO$_2$ and TiO$_2$ ENMs in both cell lines was calculated by CLS from the PSD measurements in cell culture media, see Figure 6. The CLS technique provides a weight value that corresponds to the total particle weight contained in the analysed sample, irrespective of their size. The PSD obtained from the incubation control without ENMs was subtracted from each particle weight distribution. In addition, the measurements were corroborated with respect to a control containing the applied dose without incubation (to avoid medium contributions). The results show that the SiO$_2$ ENMs deposited dose in the A549 culture medium was lower at 24 and 48 hours in comparison to the 6 and 72 hours where the deposited dose was similar. The progressive deposited dose decrease was less marked in the Epilife culture medium. For TiO$_2$ ENMS, their behaviour is quite similar in both cell culture media.
Figure 5. Percentage of PPs (---) and agglomerates (— ——) from CLS measurements of a) SiO$_2$ ENMs and b) TiO$_2$ ENMs dispensed in Epilife culture medium at a 20 µg/mL nominal dose during increasing incubation times N-hTERT keratinocytes. Values for incubation time of “0 h” correspond to the water pre-dispersion before contact with the culture medium. Both dashed lines are guides to the eye.

Figure 6. Deposited dose calculated from CLS measurements of a) SiO$_2$ ENM and b) TiO$_2$ ENMs dispersions during in vitro incubations in two cell culture media: MEM plus serum for A549 cell line (---) and Epilife for N-hTERT keratinocytes (— ——) for a 20 µg/mL nominal dose. CDT stands for ‘cell doubling time’ which is 22 h for A549 cells and 31 h for n-HTERT keratinocytes, marked by a dotted line (···). Both dashed lines are guides to the eye.

The SiO$_2$ and TiO$_2$ ENMs deposited dose in both cell lines was also calculated by PIXE from the elemental weight measurement, see Figure 7. The SiO$_2$ ENMs deposited dose in the A549 culture medium increased at 24 and 48 hours, and then it went back at 72 hours to levels similar to those at 6 hours. In the case of Epilife culture medium, the SiO$_2$ ENMs deposited dose decreased with time. The TiO$_2$ ENMs deposited dose behaviour was mostly constant, except for the 6h value in the A549 culture medium. The trend observed indicates a slight reduction with time. The deposited dose calculated by PIXE represents the elemental weight measurement including contributions from particles (ENMs included), particle surface constituents released in the culture medium (wetting and dissolution), ionic species, proteins and nutrients (from the culture medium) and dead cells (if any). Also, another factor...
governing detection is the sample preparation: the ion beam used in PIXE has an average penetration depth of 63.18 ± 2.66 µm for MEM plus serum or Epilife, thus anything that has dried below is not measured by PIXE. This last point is especially important in the presence of high amounts of ENMs agglomerates, when the sample from the culture medium is being dried as multilayer.

![Image](image.png)

**Figure 7.** Deposited dose calculated from PIXE measurements of the a) SiO$_2$ and b) TiO$_2$ dispersions during *in vitro* assessment in two culture media: MEM plus serum for A549 cell line (---) and Epilife for N-hTERT keratinocytes (— —) for a 20 µg/mL nominal dose. CDT stands for ‘cell doubling time’ which is 22 h for A549 cells and 31 h for n-HTERT keratinocytes, marked by a dotted line (···). Both dashed lines are guides to the eye.

The calculated deposited doses by CLS and PIXE seem to indicate different results; nevertheless, these differences can be explained from the basis of each determination technique. In CLS, the nature of measurements is particle-based (entity with a defined and measurable volume by extinction of an optical detector and independent of the chemical signature), thus anything outside the range of the hydrodynamic measurement (like ionic species) is not measured. The nature of PIXE, an elemental weight measurement, has already been explained. For example, let us take the case of TiO2 in both cell lines, see Figures 6b and 7b. Both CLS and PIXE measurements show nearly the same deposited dose at all times. The ratio of TiO$_2$ agglomerates versus SiO$_2$ is 2 at all times (see Figure 4) and therefore, the sedimentation of agglomerates / aggregates does not play an important role in the differences between CLS and PIXE measurements. The deposited dose of SiO$_2$ in keratinocytes, compare Figures 6a and 7a, is lower for PIXE. This difference may be associated to the SiO$_2$ ENMs open pore filling by the Epilife medium causing a de-agglomeration process. This process not only induced more PPs, see Figure 5, but also a detachment or ion release only measured by PIXE. Therefore, the de-agglomeration process, related to the ENM SSA and the specific medium, is one key factor in the apparent discrepancy of the obtained deposited doses.

The influence of the CDT for each cell line is evidenced by the trend variations observed for the deposited dose, more marked in the case of SiO$_2$ in presence of A549 cell lines. These observations open the debate about the use of a stationary model, where the culture medium is not continuously supplied and the specific cell growth rate influence is highlighted.

While the general conclusion goes to the presence of agglomerates and the different SSA, more ENMs should be tested, and this will be part of a future set of studies in regard to the cell deposited dose and the dynamics of ENMs in *in vitro* assays.

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