Interference between nanoparticles and metal homeostasis

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\textbf{Abstract.} The TiO\textsubscript{2} nanoparticles (NPs) are now produced abundantly and widely used in a variety of consumer products. Due to the important increase in the production of TiO\textsubscript{2}-NPs, potential widespread exposure of humans and environment may occur during both the manufacturing process and final use. Therefore, the potential toxicity of TiO\textsubscript{2}-NPs on human health and environment has attracted particular attention. Unfortunately, the results of the large number of studies on the toxicity of TiO\textsubscript{2}-NPs differ significantly, mainly due to an incomplete characterization of the used nanomaterials in terms of size, shape and crystalline structure and to their unknown state of agglomeration/aggregation. The purpose of our project entitled \textbf{NanoBioMet} is to investigate if interferences between nanoparticles and metal homeostasis could be observed and to study the toxicity mechanisms of TiO\textsubscript{2}-NPs with well-characterized physicochemical parameters, using proteomic and molecular approaches. A perturbation of metal homeostasis will be evaluated upon TiO\textsubscript{2}-NPs exposure which could generate reactive oxygen species (ROS) production. Moreover, oxidative stress consequences such as DNA damage and lipid peroxidation will be studied. The toxicity of TiO\textsubscript{2}-NPs of different sizes and crystalline structures will be evaluated both in prokaryotic (\textit{E. coli}) and eukaryotic cells (A549 human pneumocytes, macrophages, and hepatocytes). First results of the project will be presented concerning the dispersion of TiO\textsubscript{2}-NPs in bacterial medium, proteomic studies on total extracts of macrophages and genotoxicity on pneumocytes.
1. Introduction

Titanium dioxide (TiO$_2$) nanoparticles (NPs) are manufactured nanosized materials that are already used for a wide range of applications [1]. These applications are principally related to their strong ability to absorb UV-light and their photocatalytic activity. In addition, TiO$_2$ is chemically stable and resistant to photocorrosion and has a high ability to break molecular bonds leading to degradation of many organic compounds. Compared to bulk TiO$_2$, nanosized particles are significantly more reactive due to their larger specific surface area. These properties, together with its high abundance and low cost, have generated widespread use of TiO$_2$-NPs in popular products such as sunscreens, toothpastes and cosmetics [2-4]. Moreover, TiO$_2$ is used at the nanoscale level to decontaminate air, soil, and water by destruction of microorganisms and pesticide [5-8]. The proliferation of applications and products containing TiO$_2$-NPs will inevitably result in release into the environment and may cause health problems to human beings.

Therefore, the potential toxicity of TiO$_2$-NPs on human health and environment has attracted particular attention. Despite the large number of studies on the toxicity of TiO$_2$-NPs, results are often contradictory, certainly because the tested nanoparticles differ in their physico-chemical characteristics or in their agglomeration status. Since these characteristics are often not related in the articles, reported results are difficult to compare. First studies have revealed induction of inflammatory responses, cytotoxicity, and reactive oxygen species (ROS) formation in a variety of cell types and tissues [9-15]. These studies have identified either size or crystalline phase as the key determinant for TiO$_2$-NPs toxicity and suggest a larger toxicity of the smaller NPs in anatase cristalline phase. By contrast, other studies have shown no induction of toxic event following TiO$_2$-NPs exposure [16,17]. However, accurate characterization of NPs (e.g. chemical composition, size, morphology, surface area and crystallinity) has not always been described and it seems still essential to establish the relationship between properties of NPs and their toxic potential to biological systems and to ensure that results are reproducible and relevant [18-22]. Many of these characteristics can be determined using dry, powdered NPs but aggregation of TiO$_2$-NPs in culture media and salted buffers should also be studied. Indeed, NPs have been shown to agglomerate immediately upon addition to cell culture media that would result in inaccurate results of toxicity [23-25]. Hydrodynamic size of NPs dispersed in liquids is thus often larger than the nominal primary size of the particles. Based on these considerations, increasing attention is being paid to optimize the dispersion and the stability of suspensions of TiO$_2$-NPs. Toward this objective, several studies have investigated the influence of different types of dispersing agents to favour the slurrying of TiO$_2$-NPs in solution and to produce stable suspensions [23,26-28].

Several studies have suggested that toxic effects of TiO$_2$-NPs may be related to the generation of ROS, leading to damage on cellular and subcellular structures [10,11,29-32]. Elevated amounts of free radicals in cells exposed to TiO$_2$-NPs have also been shown to induce oxidative DNA damage [33,34]. Despite these clear effects identified in cells treated with TiO$_2$-NPs, the precise mechanisms of toxicity and their consequences on the metabolism and adaptation are largely unknown. The interrelationship between disruptions in metal homeostasis (primarily iron and copper) and increased oxidative damage have been demonstrated in both prokaryotic [35] and eukaryotic cells [36].

For example, iron is a trace element of crucial importance to living cells that is redox active and may exist into mainly two oxidative states, ferrous and ferric ions. Iron is essential to many metalloenzymes and metalloproteins involved in vital biological processes (oxygen transport, energy production, respiratory process, DNA biosynthesis, and xenobiotic metabolism...), that require electron transfers. However, excess of available iron can result in a number of cellular disturbances such as oxidative stress because of its propensity to react with oxygen, generating harmful free radicals via the Fenton reaction. Because of this dual nature, control of iron homeostasis is critical for cell survival and should be strictly regulated in relation with oxidative stress responses.

Because of the above drawbacks, transition metals levels are tightly regulated by metal homeostasis to maintain the appropriate amount of metals in the cell. Oxidative stress represents an unbalance
between the production of ROS and the potency of antioxidant defenses. The enzymes that detoxify ROS often are metalloenzymes. In addition oxidative stress conditions and metal homeostasis dysfunction may cause metalloenzymes inactivation or breakdown in endogenous antioxidant defences with potential relevance for the toxicity of some NPs.

Therefore, exposure to TiO$_2$-NPs may lead to disruption of metal homeostasis and oxidative stress that are closely related.

### 2. Description of the project

The NanoBioMet project will focus on the interference between oxidative stress and changes of metal homeostasis induced by TiO$_2$-NPs exposure in both prokaryotic and eukaryotic cells (figure 1). The toxicity of several TiO$_2$-NPs either synthesized by laser pyrolysis (N. Herlin-Boime and coworkers) or purchased from manufacturers such as Degussa or Sigma-Aldrich will be studied. Laser pyrolysis technique produces particles of high chemical purity, low mean sizes and remarkable narrow size distributions [37].

Different groups of the Commissariat à l’Energie Atomique et aux Energies Alternatives (CEA) with complementary competences are involved in this project. The key factors taken into account for this project will be:

- synthesis by laser pyrolysis of TiO$_2$-NPs then physico-chemical characterization and state of agglomeration/dispersion status of the NPs in cell culture media;
- effects of TiO$_2$-NPs on *Escherichia coli* prokaryotic cells: iron homeostasis, siderophore (ferric ion specific chelating agents) synthesis, enzymatic activities of iron-sulfur proteins and oxidative stress enzymes in *E. coli*;
- effects of TiO$_2$-NPs on several types of eukaryotic cells (macrophages, pneumocytes and hepatocytes): global stress response by proteomic approaches, iron and copper homeostasis, activation of the Unfolded Protein Response (UPR), oxidative stress enzymes, genotoxicity and DNA damages.

**Figure 1.** Schematic representation of the objectives of the NanoBioMet project.

### 3. Preliminary results

#### 3.1. Characterization of TiO$_2$-NPs and dispersion in aqueous solution

The morphology, the primary size, the specific surface area and the crystal form of two nanoparticle powders have been characterized (table 1). The commercial DegusaP25 is a widely used industrial photocatalyst while CEA12A has been synthesized at the CEA by laser pyrolysis. Both NPs are spherical. The mean primary diameter of CEA12A-NPs is 12±3 nm while DegussaP25 are twice larger. On the contrary, specific surface area is higher for the CEA12A-NPs. Finally, crystalline structure of CEA12A-NPs is 95% Anatase and this percentage is lower for DegussaP25 with 75%.
Table 1. Nanoparticles physico-chemical characteristics.

| Supplier | Morphology | Size (TEM) | SSA (m²/g) | Crystalline structure |
|----------|------------|------------|------------|-----------------------|
| P25      | Degussa    | spherical  | 25±7 nm    | 46±1                  | 75% anatase           |
| 12A      | CEA        | spherical  | 12±3 nm    | 82±4                  | 95% anatase           |

Size and morphology were determined by transmission electron microscopy (TEM, Philips EM208, accelerating voltage 80 kV). Specific surface areas (SSA, m²/g) were measured using Brunauer, Emmet and Teller (BET) method consisting in isothermal gas-adsorption [38] using a Micromeritics Flowsorb 2300 (Norcross, USA). Crystalline structure was determined by X-ray diffraction (XRD).

The dispersion of NPs in aqueous suspension is also a prerequisite for the adequate assessment of their potential biological effects. Ultrasonication is a commonly used technique to prepare highly dispersed suspensions [27,39]. Suspensions of DegussaP25-NPs (10 g/L) were prepared in ultrapure sterile water. Then the influence of different ultrasonication methodologies on the resultant hydrodynamic size of particles was investigated. Two types of probes were compared on the same sonicator (Autotune 750 W, Bioblock Scientific, Illkirch, France): a titanium tapered microtip (3 mm Ø) and a Cup Horn device (64 mm Ø). The microtip, which produces ultrawaves, is in direct contact with the solution. With the Cup Horn sonicator, the suspension is instead kept in tubes that are put into a water bath through which the waves are transmitted. Therefore, the Cup Horn delivers high energy indirect sonication: it avoids exposure of the solution to a titanium probe and provides a level of containment of NPs. All sonications were carried out at 4°C, using a pulsed mode and a circulating water bath. Two sonication times have been tested: 15 and 30 min.

Characterization of the particle size distribution was performed by Dynamic Light Scattering (DLS, Dynapro™ NanoStar, Wyatt Technology). This technique analyzes, through photon correlation spectroscopy, fluctuation with time of the particles light scattering intensity. Dynamics software (Wyatt Technologies Corp.) was used to calculate and fit an autocorrelation function plotted from the random fluctuations in scattering intensity. The intensity autocorrelation functions before and after sonication either with tapered microtip or a Cup Horn device, are presented (figure 2). The scattered light can be related to the particle translational diffusion coefficient (Dt). Finally, the hydrodynamic diameter (dH) of the NPs, which corresponds to the diameter of a sphere that has the same diffusion coefficient as the particles, can be estimated using the Stokes-Einstein equation dH = kT/3πηD; where k is the Boltzmann’s constant, T is the absolute temperature, and η is the viscosity of the solvent. Comparison of results was made with another DLS instrument: the ZetaSizer 3000HS (Malvern, Worcestershire, UK). The correlation functions from DLS were analyzed by the constrained regularized CONTIN method. For both apparatus, the mean size for the sample and the size of major population were determined.

Table 2. NPs sizes obtained after 30 min of ultrasonication with either a microtip or a Cup Horn device. Measurements of NPs in ultrapure sterile water (10 g/L) were carried out using either the Dynapro (Wyatt) or the Zetasizer (Malvern).

| Microtip | Cup Horn | Microtip | Cup Horn |
|----------|----------|----------|----------|
| Dynapro (Wyatt) | 159 | 163 | 183 | 162 |
| ZetaSizer (Malvern) | 128 | 129 | 46 | 23 |
Table 2 presents the summary of the results obtained for DegussaP25. After 30 min of sonication, the particle size measured with the Wyatt instrument was about 160 nm using either the microtip or the Cup Horn device. Previous articles [27,40] reported similar sizes after ultrasonication of DegussaP25 NPs in aqueous suspension. To obtain these results with the Cup Horn, optimization has been done: a water bath circulating system was used to avoid overheating and a 12 mL tube was put in the centre of the Cup Horn to increase the sonication energy. It can be concluded that the use of a Cup Horn sonicator seems a useful way of dispersing nanoparticles in aqueous suspension.

Measurements were also carried out with the Zetasizer from Malvern on the same NPs solutions. However, the sizes measured with this second apparatus were different. This may be explained by the fact that the same raw data may be treated in different ways by the software packages provided with the two DLS instruments. Therefore, comparison of size data obtained with the two DLS instruments is difficult.

![Intensity autocorrelation functions of TiO$_2$-NPs suspensions in water before sonication or after 30 min of sonication either with tapered microtip or a Cup Horn device (Autotune 750 W, Bioblock Scientific). The concentration of suspensions was 10 g/L.](image)

**Figure 2.** Intensity autocorrelation functions of TiO$_2$-NPs suspensions in water before sonication or after 30 min of sonication either with tapered microtip or a Cup Horn device (Autotune 750 W, Bioblock Scientific). The concentration of suspensions was 10 g/L.

3.2. Effects of TiO$_2$-NPs on E. coli growth
Before studying the effects of NPs on bacterial growth, NPs (DegussaP25 and CEA12A) suspension stability was evaluated in bacterial media. Indeed, the objective is to select a medium allowing bacterial growth but avoiding NPs aggregation. In commonly used bacterial media, the Luria-Bertani
Broth (LB) rich medium and the M9 minimal medium, TiO$_2$-NPs showed a strong aggregation. Therefore, a minimal medium containing low concentration of salts and allowing bacterial growth was optimized. It consisted of: 0.4% Glucose, 50 µM MgSO$_4$, 0.1% casamino acids. Nevertheless, NPs aggregation was still observed in this medium. Therefore, a dispersing agent sodium pyrophosphate (Na$_4$P$_2$O$_7$), pH 7.0, was added. It increased NPs surface charge and the electrostatic repulsive force between particles (Jiang et al, 2009). Although this agent was not able to prevent aggregation in LB and M9 media, it efficiently prevented NPs aggregation in this optimized minimal medium. No increase in the NPs size in this culture medium was observed compared to stock solution in water and this solution was stable for several days.

![Graph showing the effects of TiO$_2$-NPs on growth of *E. coli* MG1655](image)

Figure 3. TiO$_2$-NPs effects on growth of *E. coli* MG1655. Cultures were grown in medium consisting in 0.4% Glucose, 50µM MgSO$_4$, 0.1% casamino acids, 5mM Na$_4$P$_2$O$_7$ pH 7. NPs were added at t=0 at final concentrations of 10 or 100 µg/mL. OD$_{600}$ were recorded.

The effects of TiO$_2$-NPs (DegussaP25 and CEA12A) at concentrations of 10 and 100 µg/mL on the growth of *E. coli* were then evaluated (figure 3). The growth rates were measured at 37°C by monitoring the optical density at 600 nm in a microplate reader (Tecan Infinite F200, Männedorf, Switzerland). Surprisingly, no decrease in bacterial growth was noticed following exposure to 10 or 100 µg/mL of both NPs. An increase was even observed after exposure to 100µg/mL. On the contrary, a previous study showed that survival of *E. coli* was affected when exposed to these TiO$_2$-NPs [41]. However, these experiments have been done in water which could explain the differences with our study.

3.3. Effects of TiO$_2$-NPs on murine macrophages

Macrophages play a major role in eliminating foreign matter, e.g. pathogens or infectious agents, by phagocytosis and inflammatory responses, and are thus highly likely to react to NPs. As a model system, we have utilized macrophages derived from murine bone marrow [42]. Mice were killed by cervical dislocation and bone marrow was obtained by flushing femura and tibiae with BM20 medium (DMEM supplemented with 10% FCS, 20% L929 cell-conditioned medium as a source of M-CSF, 5% horse serum, 1% glutamine and 1% sodium pyruvate). Before adding TiO$_2$-NPs on the macrophages, flow cytometry was used to check if cells were mature macrophages, expressing both CD11b and CD14. Cells (5x10$^6$) were cultured in Petri dishes and treated for 24h with 100 µg/ml of TiO$_2$ (anatase,<25 nm from Sigma-Aldrich) diluted in polyvinylpyrrolidone 40 (PVP40) 0.2% that increased the dispersion of NPs but did not prevent their aggregation.

Proteomic analysis based on 2D gels was used and performed on whole cell extracts. This study revealed that TiO$_2$-NPs induced an increase in the expression of proteins involved in different
metabolic pathways, some of them related to the iron homeostasis control and oxidative stress response (See communication by M Chevallet et al. for more details about the all set of proteins increasing in expression upon NPs treatment). Indeed, an induction of peroxiredoxin 4 and ferritin (light chain) was observed. Peroxiredoxins (Prxs), a family of thioredoxin-dependent peroxidases, are known to protect aerobic organisms against oxidative stress by degradation of peroxides and other substrates [43]. Mammalians ferritins are composed of 24 subunits that form an almost spherical shell, delimiting a large cavity that can accommodate up to 4000 Fe atoms. They are known to play a role in the regulation of available cellular iron and storage in the response to oxidative stress. These preliminary results suggest that the effects of TiO\textsubscript{2}-NPs may be mediated through oxidative stress and a putative modification of iron homeostasis. Further experiments have to be done with characterized and dispersed solutions of DegussaP25 and CEA12A NPs in order to see if they trigger the same changes in ferritin and peroxiredoxin contents.

3.4. Cytotoxicity and genotoxicity of TiO\textsubscript{2}-NPs to A549 human pneumocytes
Toxicity of TiO\textsubscript{2}-NPs was evaluated in pneumocytes since NPs might be inhaled by populations and reach pulmonary alveoli. To model this situation, we studied the response of A549 human pneumocytes after exposure to DegussaP25-NPs diluted in DMEM medium supplemented with 50 UI/mL penicillin and 50 µg/mL streptomycin. Cell metabolic activity, reflecting toxicity of NPs, was evaluated by using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) reduction method [44]. Mitochondrial dehydrogenases of viable cells reduce MTT to water insoluble blue formazan crystals which are then solubilised by dimethyl sulfoxide (DMSO). Therefore, this assay indicates cell mitochondrial activity impairment. After exposure to 100 µg/mL of TiO\textsubscript{2}-NPs, the cell death rate measured with the MTT assay was 17% and 48% after 48h and 72h, respectively.

To determine whether these observation could be explained by induction of oxidative stress, the formation of ROS was measured via monitoring the increased fluorescence of 2',7'-dichlorodihydrofluorescein diacetate acetyl ester (H\textsubscript{2}DCF-DA, Invitrogen [45]) (figure 4). This hydrophobic non-fluorescent chemical can get across the cell membrane easily, in order to be converted into hydrophilic non-fluorescent form by esterases which remove acetate groups. This compound is oxidized by intracellular ROS and turned into hydrophilic green fluorescent form, DCF. Fluorescence count was significantly higher than in the negative control after exposure to 100 µg/ml of TiO\textsubscript{2}-NPs. It was about 6 times higher than control after both 4h and 24h of exposure.

![Figure 4. Fluorescence count per µg of proteins quantified in A549 human pneumocytes exposed to TiO\textsubscript{2}-NPs at 100 µg/mL after 4h or 24h of exposure.](image)

After showing the occurrence of oxidative stress upon incubation with NPs, we studied its potential genotoxic properties. We thus first quantified the level of 8-oxo-dG formation as a biomarker of oxidative damage of DNA after ROS production. The level of 8-Oxo-7,8-dihydro-2′-deoxyguanosine (8-oxo-dG) was measured in cells exposed to TiO\textsubscript{2}-NPs using high-performance liquid
chromatography coupled to tandem mass spectrometry (figure 5). Production of 8-oxo-dG residues in DNA leads to a GC→TA translocation unless repaired prior to DNA replication [46]. Therefore, the presence of 8-oxo-dG may lead to mutagenesis. Furthermore, many observations indicated a direct correlation between in vivo 8-oxo-dG formation and carcinogenesis [47,48]. The percentage of 8-oxo-dG was statistically higher for cells exposed to TiO₂-NPs, which is a sensitive marker of ROS-induced DNA damage.

Finally, the comet assay was performed under alkaline conditions to assess NPs-induced DNA damage [49]. The comet assay (single cell gel electrophoresis) detects DNA strand breaks and alkaline labile sites in individual cells. These classes of DNA lesions mostly result from oxidative processes. At least 50 comets per sample were analyzed under a fluorescence microscope. The comet tail length was increased by a factor 4.3 and 11.6 as compared to unexposed cells following 4h and 24h of exposure, respectively. These results indicated that TiO₂-NPs caused DNA breakage. The genotoxicity may come from direct interaction between NPs and DNA or through the attack of DNA by over-accumulated ROS, which may gain access to cell nucleus by diffusion through nuclear pores and then attack DNA.

4. Conclusion
These first results confirmed the importance of the NPs characterization (size, stability…) after dispersion in culture media in order to obtain reproducible and meaningful results. We obtained TiO₂-NPs dispersion in a minimal bacterial growth medium that we optimized. Therefore, we will be able to follow modification of protein expression and enzymatic activity of metalloenzymes caused by both NP-TiO₂ (CEA12A and DegussaP25) studied in this article, even if no effect on the bacterial growth was observed in the tested conditions. Another experiment done for the NanoBioMet project revealed that aggregated TiO₂-NPs (anatase, <25 nm, Sigma-Aldrich) induced an increase in the expression of proteins including a ferritin subunit and a peroxiredoxin in macrophages. Finally, toxicity of DegussaP25-NPs was evaluated in A549 pneumocytes. Decrease in viability, formation of 8-oxo-dG, DNA strand breaks and ROS, were observed suggesting oxidative stress conditions upon TiO₂-NPs treatment. Further work is currently in progress in order to identify and quantify putative metallomic perturbations following treatments of various cellular models by well-characterized NPs.

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