Endocytosis, oxidative stress and interleukin-8 expression in human lung epithelial cells upon treatment with titanium dioxide particles: role of particle surface area and surface methylation

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[Toxicology and Applied Pharmacology, 2007, 222, 141-151 DOI: 10.1016/j.taap.2007.05.001]

ENDOCYTOSIS, OXIDATIVE STRESS AND INTERLEUKIN-8 EXPRESSION IN HUMAN LUNG EPITHELIAL CELLS UPON TREATMENT WITH TITANIUM DIOXIDE PARTICLES: ROLE OF PARTICLE SURFACE AREA AND SURFACE METHYLATION

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ABSTRACT

Inhaled ultrafine particles show considerable stronger pulmonary inflammatory effects when comparatively tested at equal mass dose with their fine counterparts. In line with this, a number of in vitro studies have demonstrated that such ultrafine particles elicit an enhanced release of pro-inflammatory cytokines, but the exact mechanisms whereby these effects may occur are not yet fully understood. The aim of this study was to investigate the role of particle size and surface chemistry in initiating pro-inflammatory effects in vitro using the A549 human lung epithelial cell line on treatment with different model TiO_2 particles. The role of surface area and surface properties was investigated by comparing the responses of two samples of TiO_2, i.e. fine (40-300nm) and ultrafine (20-80nm), both in their native forms as well as upon surface methylation. Methylation of the samples was confirmed by Fourier Transformed Infrared Spectroscopy (FR-IR). The radical generating properties The generation of radicals of the particles in the cell cultures was determined by means of electron paramagnetic resonance (EPR) with by using 5,5-dimethyl-1-pyrroline-N-oxide (DMPO) or 4-Hydroxy-2,2,6,6-tetramethylpiperidine-1-oxyl (TEMPOL) as spin trapping agents. The expression and release of the pro-inflammatory chemokine interleukin-8 (IL-8) was determined by RT-PCR and ELISA respectively, whereas particle uptake was determined by transmission electron microscopy. TiO_2 particles were rapidly taken up by the A549 cells and generally observed as membrane bound aggregates as well as large intracellular aggregates in vesicles, vacuoles and lamellar bodies. Aggregate sizes tended to be smaller for the ultrafine samples, and were also smaller for methylated fine particles when compared to non-methylated fine particles. No particles were observed inside the nucleus or any other vital organelle. Both ultrafine TiO_2 samples but not their fine counterparts elicited significant stronger oxidant generation and IL-8 release from A549 cells, despite their high aggregation state and irrespective of their methylation. In conclusion, present data indicate that ultrafine TiO_2, even as aggregates/agglomerates trigger inflammatory responses that appear to be driven by their large surface area. Further studies are needed to determine whether these effects are mediated by oxidative stress as resulting from particle-cell interactions rather than from a direct, intrinsic, surface reactivity of the particles.

Key words - Titanium dioxide, oxidative stress, ultrafine particles, nanoparticles, inflammation, lung epithelial cells, endocytosis

INTRODUCTION

Particles less than 100nm in diameter, which are usually referred to as ultrafine particles by toxicologists and as nanoparticles by physicists, have been considered to be important in driving the adverse health effects of particulate air pollution (Peters et al., 1997; Utell and Frampton, 2000; Oberdorster et al., 2005b; Donaldson et al., 2005). Experimental support for this hypothesis has been predominantly provided from studies in rats with commercially available model particles such as carbon black and titanium dioxide, where such model particles in the ultrafine size range gave markedly stronger inflammatory and toxic effects in the lungs of experimental animals when compared to their non-ultrafine counterparts (Ferin et al., 1992; Li et al., 1999; Oberdörster 2001; Höhr et al., 2002). Taken together with *in vitro* findings with such materials (e.g. Brown et al., 2004), these studies have raised questions about the possible adverse health implications for large scale uncontrolled commercial production of nanoparticles (Colvin, 2003; Borm and Kreyling, 2004; Oberdorster et al., 2005a). Notably, both carbon black and titanium dioxide have recently been (re)classified as possible human carcinogens (group 2B) by the International Agency of Research on Cancer, which was partly supported by mechanistic considerations on the importance of particle-induced chronic inflammation in tumourigenesis in rat inhalation studies (Baan et al., 2006).

Concerning the mechanism of toxicity of nanoparticles, it has been suggested that the particle specific surface area and/or its chemical composition are major determinants (Brown et al., 2001; Nel et al., 2001; Donaldson et al., 2002). A wide variety of low toxicity poorly solubility particle types have been shown to induce inflammation proportional to their surface area exposed, indicating suggesting that large amounts of surface area particles alone in the lung may be sufficient to initiate inflammation (Duffin et al., 2002; Schulz GSF CB paper). One hypothesis to explain these surface area related effects is the generation of cellular oxidative stress leading to the activation of redox sensitive signaling pathways that culminate in the transcription of pro-inflammatory cytokines and chemokines (Donaldson et al., 2002; Oberdorster et al., 2005b). In support of this hypothesis, several types of ultrafine particles have been shown to elicit stronger oxidising properties than their fine counterparts, if subjected to acellular assays such as plasmid DNA unwinding/breakage or fluorescence dye oxidation if compared on a per weight basis??? (Donaldson et al., 1996; Brown et al., 2001; Dick et al., 2003). Notably however, apart from direct oxidant generation, particles can also generate cellular derived ROS and oxidative stress in an indirect manner, i.e. resulting from their interactions with target cells such as a response of macrophages and epithelial cells to

particle interaction. Indeed, several particulate materials including asbestos, quartz, coal dust or ambient particulate matter have been shown to generate ROS via processes involving activation of NAD(P)H-like enzyme systems or via modulation of mitochondrial respiration (Fubini and Hubbard, 2003; Knaapen et al., 2005; Upadhyay et al., 2003; Nel et al., 2003). However, the significance of this indirect, cell-mediated ROS generation for the observed contrasts in fine versus ultrafine particle effects, is currently still poorly addressed.

Among the best investigated material in current nanotoxicology research is titanium dioxide. Non-ultrafine, pigmentary grade TiO_2 has seen wide applications e.g. as paint filler, food additive or in sunscreen formulations, and has been incorporated as negative control in many particle toxicology studies. In contrast, ultrafine TiO_2 has been shown to elicit toxic and inflammatory effects in a variety of *in vitro* and *in vivo* studies. Hallmark studies by Oberdorster and colleagues demonstrated that ultrafine TiO_2, when instilled intratracheally into rats and mice, induced a much greater pulmonary-inflammatory response than fine TiO_2 at the same instilled mass dose (Reviewed in Oberdorster et al., 2005b). More recent studies also showed that nanosized TiO_2 in contrast to larger size TiO_2 can be highly photoreactive in presence of UV light (Serpone et al., 2001), and such mechanism has been considered to drive free radical mediated toxicity and DNA damage in skin cells (Dunford et al. 1997). Notably however, in the absence of photosensitisation, ultrafine TiO_2 has also been shown to elicit comparatively stronger plasmid DNA unwinding than fine TiO_2 (Donaldson et al., 1996), although this was not observed in subsequent studies (Dick et al., 2003). Taken together, many studies nowadays suggest that TiO_2 in the nanosize range represents a potentially toxic material. Notably however, in contrast to many engineered nanoparticles, TiO_2 typically occurs, and hence ineracts as aggregates of particles in biological environment (Stearns et al., 2001; Rehn et al., 2003). Furthermore, many commercial forms of TiO_2 contain coated surfaces, a procedure which is usually applied with engineered nanoparticles to improve their physicochemical properties. Such aspects of aggregation and agglomeration, as well as surface coatings of nanoparticles are very likely to affect their interactions with cells and subsequent toxic stress responses. Previously, we investigated the the inflammatory effects of native versus surface methylated preparations of fine and ultrafine TiO_2 after their intratrachal instillation in rat lungs. The effect of size and of surface methylation was investigated by some of us in a previous study by monitoring the effect on instillation in rat lungs of fine and ultrafine TiO2, as prepared or surface methylated. These studies Such study showed, in line with other studies, a stronger inflammatory toxicity for the ultrafine particles compared with

their fine counterparts, whereas while the effect of methylation on both samples was negligible (Höhr et al., 2002).

In order to gain further insight in the mechanisms whereby these contrasting effects occured, we investigated the inflammatory properties of the same TiO_2 samples *in vitro*, in relation to their (1) primary particle size distribution, (2) aggregation/agglomeration state, (3) hydrophobicity and (4) radical generating propertiespotential. Therefore, A549 human lung epithelial cells were treated with either fine or ultrafine TiO_2 in their native or methylationed forms, and evaluated for the expression and release of the chemokine interleukin-8 by RT-PCR and ELISA, respectively. Transmission electron microscopy was used to determine particle endocytosis and state of aggregation status of the different particle in the different samples. whereas Their radical inducing or generating capacities of the different samples in A549 cell cultures were was determined using by means of electron paramagnetic resonance (EPR) with the spin trapping tecniques.

METHODS

Titanium dioxide samples

Two commercial samples, representing fine TiO_2 and ultrafine TiO_2 products, were selected for the present study. Details of the samples Nature and physical characteristics of the powders are provided reported in table 1. Both samples were of a high cationic purity. As for anions, the fine TiO_2 contained traces of sulphate species (< 0.005 wt%), while in the ultrafine TiO_2 a higher amount of chloride impurities (<0.08 wt%) was present. Such level of Cl^- ions does not affect the Lewis surface properties of stoichiometric TiO_2, as resulting from a study on a similar Cl-free TiO_2 powder (Morterra et al., 1980)

Table 1. Characteristics of fine and ultrafine TiO_2 samples.

	Specific surface area ($m^2 \cdot g^{-1}$)	**Size range (nm)**	**Composition**	**Source/name**
Fine	10	40-300	pure anatase	Merck
Ultrafine	50	20-80	80% anatase, 20% rutile	Degussa P25

Both samples were subjected to a methylation procedure in order to change the substantially hydrophilic surface of these particles into a more hydrophobic one. Thus, a total of four different TiO_2 samples was applied in the present study, i.e. the native forms of fine titanium dioxide (F-TiO_2) and ultrafine titanium dioxide (UF-TiO_2), as well as the methylated forms of fine titanium dioxde (MF-TiO_2) and ultrafine titanium dioxide (MUF-TiO_2). The same samples have previously also been used in an *in vivo* study by our group (Höhr et al., 2002).

Methylation of titanium dioxide particles

For this procedure both samples were outgassed at room temperature for 45 min in a conventional vacuum line, under a residual pressure of 1.0×10^{-6} Torr (1 Torr= 133.33 Pa); the outgassing temperature was then raised up to 250 °C and kept at such a value for 45 min, to remove most part of water molecules adsorbed on the TiO_2 surface without significant loss of surface hydroxyl groups by condensation. Subsequently, CH_3OH was added (40 torr) and the samples were kept in contact with methanol for 45 min at 250 °C and finally outgassed at room temperature for 45 nim. The efficacy of methylation of the TiO_2 dusts was investigated

by Fourier Transformed Infrared Spectroscopy (FTIR). Therefore, the TiO_2 powders were pressed in the form of self-supporting pellets (*ca.* 20 $mg \cdot cm^{-2}$ and placed in a conventional IR quartz cell equipped with KBr windows, permanently connected to the a vacuum line, allowing all thermal adsorption-desorption experiments to be carried out *in situ*.

Evaluation of endotoxin contamination

The four different TiO_2 samples used in the present study, were also analysed for possible endotoxin contamination. Therefore, the four different particle preparations, were suspended in endotoxin free water, and then subjected to a quantitative kinetic chromogenin Limulus Amoebocyte Lysate (LAL) method (Bio Witthaker), using Escheria coli 055:B5 endotoxin (Bio Witthaker) as standard.

Culture and treatment of A549 human lung epithelial cell line

In the present study, we used A549 human lung epithelial cells. This cell line has been proved to be a suitable *in vitro* model to study endocytosis by ultrafine particles including TiO_2 (e.g. Stearns et al., 2001), as well as to investigate the inflammatory potential of various particles (Simeonova and Luster, 1996; Schins et al., 2000). A549 cells (American Type Culture Collection/ATTC), were grown in Dulbecco's Modified Eagle's Medium (DMEM; Life Sciences), supplemented with 10% heat inactivated fetal calf serum (FCS; Life Sciences), L-glutamine (Life Sciences), and 30 IU/ml penicillin-streptomycin (Life Sciences) at 37°C and 5% CO_2. For experiments, cells were grown until near confluency (90-95%) in complete culture medium. The medium was then replaced with FCS-free medium for 24 hours until treatment. Immediately before the start of the incubations, particle suspensions were prepared in culture medium or HBSS and sonicated for 5 minutes using a water bath (Sonorex TK52 water bath; 60W, 35kHz). A549 cells were rinsed twince with PBS and then immediately treated with the particle suspensions at the indicated concentrations and incubation time intervals. The respirable quartz standard sample DQ12 (9.6 $m^2 \cdot g^{-1}$, Batch 6-IUF) and Tumour Necrosis Factor alpha (TNF-α) were used as particulate and non-particulate positive controls, respectively (Schins et al., 2000; Fiedler et al., 1998).

Analysis of particle uptake in A549 cells by transmission electron microscopy

A549 cells were grown in complete culture medium in 35 mm culture dishes until 90-95% confluency, and then cultured for a further 24 hrs in serum free medium. The cells were then washed twice with PBS and treated with 16 or 80 $\mu g/cm^2$ native or methylated TiO_2 for 4h in

complete medium. Following incubation, the cell monolayers were immediately rinsed three times with serum free medium and then fixed with 2% glutaraldehyde in 0.1 M sodium cacodylate buffer for 1 h at 4°C. After post-fixation in 2% OsO4 in 0.1 M sodium cacodylate buffer for 1 h at 4°C, the samples were en bloc stained with 1.5% uranylacetate dihydrate and phosphotungstic acid, dehydrated in ethanol series and embedded in Epon epoxy resin (Serva, Heidelberg, Germany). Morphologic characteristics of the cells and the distribution and agglomeration state of the particles within the cells were investigated using ultra thin sections (50 nm) placed on 150 mesh grids and examined by transmission electron microscopy (STEM CM12, Philips) in combination with a digital imaging system (SIS, Münster, Germany). EDX-analysis and elemental mapping was used for identification of the TiO_2 samples.

Analysis of reactive oxygen species formation by electron paramagnetic resonance (EPR) analysis with the spin-trapping technique.

Electron paramagnetic resonance (EPR) spectroscopy in combination with spin-trapping agents is a technique used extensively to detect and identify many short-lived free radical compounds, such as superoxide and hydroxyl radicals, in biological systems. With this approach we determined the formation of reactive oxygen species by the different preparations of TiO_2. Two different spin-trapping agents were used, i.e the spin-trap 5,5-dimethyl-1pyrroline-N-oxide (DMPO), which gives stable radical adducts with small free radicals, and the spin-probe TEMPOL (4-Hydroxy-TEMPO 4-Hydroxy-2, 2, 6, 6 tetramethylpiperidine-1-oxyl), a stable radical which is progressively blunted upon contact with radicals.

The generation of radicals generating properties of the different particle preparations during the treatment of the A549 cells with the different particle preparations were was determined as follows. Cells were grown as described before, starved for 24 hours using serum free medium, rinsed twice with PBS and then immediately treated with the different particle samples. All TiO_2 samples were freshly suspended in HBSS at 0.8 mg/ml, sonicated for 5 minutes (Sonorex TK52 water bath; 60W, 35kHz) and then 450 µl of these suspensions were immediately added to the cell culture dishes. Following 1h incubation either 50µl of 0.05 mM TEMPOL or 1M DMPO (in PBS) was added to the cell cultures for 1h or 3h. Control incubations were performed for both spin-trapping agents in the absence of the various particle samples and/or A549 cells. Supernatants were collected at the indicated time points, briefly vortexed, immediately transferred into a 50µl glass capillary and measured with a Miniscope MS100 EPR spectrometer (Magnettech, Berlin, Germany). The EPR-spectra

were recorded at room temperature using the following instrumental conditions: Magnetic field: 3360 G, sweep width: 100 G, scan time: 30 sec, number of scans: 3, modulation amplitude: 1.8 G, receiver gain: 1000 the maximum for miniscope is not 900?, microwave power ? For the experiments performed with DMPO, which is known to react with both ·OH and $O_2^{\cdot -}$ to form products with different spectra , experiments in the absence of A549 cells were also performed in the presence of ethanol to determine the specificity of the obtained spectrum for ·OH. if the signal was due to hydroxyl or superoxide radicals. The adduct of DMPO with superoxide radical is in fact known to rapidly decay to DMPO/HO· (Rif. e.g. E. Finkelstein, G.M. Riosen, E.J. Rauckman *Mol. Pharmacol.* **1982,** *21*, 262-265)

Note that the experimental conditions chosen are far from what usually employed to detect free radicals in cell-free suspensions of particles (Fubini B., Mollo L. and Giamello E. (1995) Free radical generation at the solid/liquid interface in iron containing minerals. *Free Rad. Res*. **23,** 593-614. , Fenoglio, I., Prandi, L., Tomatis, M., Fubini, B. (2001) Free radical generation in the toxicity of inhaled mineral particles: the role of iron speciation at the surface of asbestos and silica. *Redox Rep*. **6**, 235-241). Larger concentrations of the spin trapping reagents and longer incubation times were used to adapt the conditions to cellular tests.

Interleukin-8 mRNA and protein expression

Human IL-8 messenger RNA (mRNA) expression was detemined by reverse transcriptase-polymerase chain reaction (RT-PCR). Total RNA was extracted from the treated A549 cells with TRIZOL reagent (Invitrogen) using the recommended protocol. The Promega Access RT-PCR System kit was used for the amplification of human IL-8 mRNA and the human housekeeping gene, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), according the manufacturer's instructions. Sequences of the primers used, and details of the RT-PCR conditions are previously described (Schins et al., 2000).

The release of IL-8 from the A549 cells was detemined by with an enzyme-linked immunosorbent assay (ELISA), as follows. After incubation with the different particle preparations, cell supernatants were collected and immediately centrifuged (5 min, 15000rpm). The resulting supernatants were stored at –20°C until measurement (PeliKine compactTM human IL-8 ELISA kit, Sanquin, The Netherlands). For each treatment, cytokine release is expressed as a percentage of the IL-8 release as measured in the control incubations.

Statistical analysis

Data are expressed as mean ± SEM unless stated otherwise. Treatment-related differences were evaluated using one-way analysis of variances (ANOVA), with Tukey LSD or Dunett post hoc comparisons. A difference was considered significant at $P<0.05$.

RESULTS

Methylation of the TiO_2 samples

The efficiency of the methylation procedure of the particles was determined by Fourier transform infrared (FTIR) spectroscopy. Representative IR spectra for UF-TiO_2 are shown in Figure 1. Spectrum (a) corresponds to TiO_2 outgassed at room temperature for 45. In this condition a full monolayer of hydroxyl groups, both vibrationally "free" (i.e. not interacting through hydrogen bonding with neighbour OH) and or hydrogen bonded, and as well as water molecules coordinated onto surface Ti^{4+} cations coordinatively unsaturated are visible. Such features the ensemble representing the first hydration layer of the materials, which, in the experimental conditios adopted, are left on the surface. After treatment in CH_3OH and subsequent outgassing at the room temperature (spectrum b), on the one hand the bands due to H_2O molecules disappeared, because of water desorption, as well as signals due to vibrationally free hydroxyl, while a fraction of the absorptions related to H-bonded surface hydroxyls is left. On the other hand, bands due to stretching (3000-2700 cm^{-1}) and deformation (1400-1300 cm^{-1}) modes of $–CH_3$ and CO- stretching of metoxy groups (components at 1120 and 1060 cm^{-1} components, superimposed to the main band due to lattice modes of TiO_2). The overall pattern indicated that a significant part of the surface -OH groups had been replaced by $–OCH_3$ metoxy groups. Finally, water vapour (18 Torr, saturation pressure at room temperature) was admitted on the methylated sample, in order to form on the surface multilayers of physisorbed water corresponding to the interface of the material when suspended in aqueous media. The sample was thus kept in contact with H_2O vapour for 30 min and then outgassed at room temperature. This treatment was repeated until no more changes were observed in the OR spectrum, indicating that an equilibrium condition in the interactions between the methylated surfaces and H_2O molecules was reached. The corresponding IR pattern is shown as spectrum c: the bands related to methoxy groups appears decreased of ca. 50% in intensity with respect to the sample immediately after methylation, but they their presence indicates that ca. half of the organic groups substituting surface hydroxyls is still present, and apparently irreversible by upon contact with water . Of course, in addition the bands related to water irreversibly adsorbed on the surface under the repeteated contact with water outgassing at room temperature appeared again.

Endocytosis and subcellular localisation of TiO_2 particles

The endocytosis of the different paticle preparations was investigated by TEM and EDX-analysis. Representative micrographs are shown in figure 2. The TEM analysis demonstrated a rapid internalisation of the TiO_2 particles in the A549 cells. Confirmation of the identity and composition of the electron dense particles, assessed to be TiO_2, was achieved by EDX-analysis and elemental mapping. The particles, which were identified both outside and inside the epithelial cells by their specific ultra-structure at high microscopic magnification, showed the characteristic X-ray emission line of the element Ti which refers to an unequivocal identification of the particles. Over all, the TiO_2 particles were found to be associated as large aggregates and/or their agglomerates and appeared to entered the cells by phagocytosis (see Figure 2A). For the ultrafine TiO_2 particles, occasionally small aggregates containing 3-5 ultrafine primary TiO_2 particles (size 20-30 nm) were observed to be endocytosed by clathrin coated vesicles. Unclear, only UF un the pictures? Endocytosis or phagocytosis? Figure 2 has no A and B in this version.

Neither the different size of the particles nor their surface properties had an influence upon the intracellular particle distribution. Aggregates of both the fine and the ultrafine particles were predominantly incorporated in membrane-bound vacuoles. Particles aggregates were also found associated with both loosely and highly packed lamellar bodies (see Figure 2B?). Multivesiculated bodies were also found to be enriched with TiO_2 particles. Membrane-enclosed particle aggregates of different size were often observed next to the nucleus (see Figure 2D) but never inside the nucleus. Golgi apparatus, rough ER and mitochondria were all found to be free of TiO_2 particles. TiO_2 particles that were located in the space between the cells were never observed in association with moving through the tight junctions into the cells. The over all electron microscopical analyses unclear indicated that all of the above effects occurred in the absence of signs of necrosis or apoptosis.

The typical particle clustering limited the determination of the exact particle number that entered the cells. Instead, in our the present experiments we thus used the proportion of particle aggregates according to their maximal mean diameter as parameters to achieve some indications of particle uptake in relation to particle size and methylation. The effects of particle size as well as of methylation of the hydrophilic TiO_2 samples on the diameter distribution of the endocytosed aggregates is shown in figure 3. About half of the fine TiO_2 particle aggregates inside the A549 cells were found to be in the size range of 200-500 nm. In contrast, for the ultrafine particles, aggregates showed a tendency to the lower size range, i.e. <200 nm. For the fine TiO_2, the methylation procedure was associated with a reduction in the

diameters of endocytosed aggregates. However, for the ultrafine particles no such clear methylation-associated effect was observed (see Figure 3).

Electron paramagnetic resonance analysis Free radical release upon contact with cells

Electron paramagnetic resonance analysis was performed to determine the radical generation properties of the four different TiO_2 samples during treatment of in contact with A549 cells. The results of these experiments are shown in figure 4. With regard to DMPO (panel A), both UF-TiO_2 and MUF-TiO_2 showed significantly higher ROS generation, in contrast to F-TiO_2 and F-TiO_2, for which the EPR-signal intensities were not different from controls. In contrast, in the absence of cells no significant difference in signal intensities were observed among the different particle preparations. Similar observations were observed using the spin trap TEMPOL. As can been seen in (figure 5 4, panel B): using the spin-trapping agent TEMPOL, both UF-TiO_2 and MUF-TiO_2 showed a significant decrement of the signal increase ROS production after 2h as well as after 4h, in contrast to yet vosible with F-TiO_2 and MF-TiO_2. In the absence of A549 cells EPR measurements with the different TiO_2 samples in the presence of TEMPOL with both spin traps showed no differences from the control, suggesting . Although these data suggest that radicals were not generated by cells and not by the particles. itself, but rather via their interaction with the A549 cells, it should be noted Note, however, that the ESR settings were specifically optimised for measurements under cell cultures and are far form what usually employed to detect particles derived radicals. . Treatment conditions were chosen on the basis of absence of cytotoxicity with DMPO and TEMPOL, and not under conditions that would allow optimal detection of ROS generation from the particle surfaces directly.

Interleukin-8 mRNA expression and release from A549 cells

The effects of the different TiO_2 sample preparations on the expression and release of interleukin-8 from A549 human lung epithelial cells are shown in figure 5. A can bee seen in the figure, Both UF-TiO_2 and MUF-TiO_2 caused a significant increase in interleukin-8 release from the A549 cells following 24 hours treatment. This effect was not observed with F-TiO_2 and MF-TiO_2. However, tThe methylated ion state of the TiO_2 samples did not affect the production of interleukin-8. significant release of interleukin-8 expression was also observed with DQ12. When compared on equal mass basis, a significant effect of DQ12 was observed at a five fold lower concentration than for both TiO_2 sample preparations. In line with the observations on interleukin-8 release, both ultrafine UF-TiO_2 samples also caused enhanced

mRNA expression of IL-8 in the A549 cells, in contrast to both fine TiO_2 samples. Enhanced IL-8 mRNA expression was also observed after treatment with DQ12 and TNFα. Endotoxin determinations of the different dusts suspended in endotoxin free water showed values of 0.006, 0.007, 0.005, and 0.009 EU/mg dust, respectively for F-TiO_2, UF-TiO_2, MF-TiO_2, and MUF-TiO_2. These data showed that the Thus endotoxin contamination was negligible for all samples used (i.e. <0.003 EU/ml during cell treatment), and hence would not affect the outcome of our cell experiments. This was also confirmed in independent experiments where LPS failed to elicit increased IL-8 release at concentrations below 0.1µg/ml (data not shown).

DISCUSSION

Over the past years, concern has been raised about the potential harmful effects of nanoparticles (Colvin, 2003; Borm and Kreyling, 2004; Oberdorster et al., 2005a, Nel et al 2006). However, most of the toxicological data that have contributed to this debate are available from studies with a limited number of ultrafine particles, such as TiO_2. Although particle size, and more closely, the total particles surface exposed area per se, has been shown to relate to their in vitro and in vivo toxicity (Oberdorster et al., 2005a), detailed molecular mechanisms explaining for the effects as occuring are still incompletely understood. Elucidation of the responsible mechanism(s) is of major importance, since this allows for potential identification of unifying factors that may be applied in initial hazard screening strategies of novel nanomaterials (Oberdorster et al., 2005b). Important aspects of the toxicity of nanoparticles, include their surface chemistry and aggregation state and, most importantly, their their ability to generate oxidative stress (Donaldson et al., 2002).

In the present study, we evaluated, the ability of TiO_2 to induce the expression of interleukin-8 in A549 human lung epithelial cells, in relation to their exposed surface area, surface composition, aggregation/agglomeration state and ability to generate radicals and oxidative stress. The effect of surface area per se was determined by comparing the effects of two samples of TiO_2 with different particle size distribution and hence surface area. A methylation procedure, was used to change the hydrophobicity of the particle surface, The efficiacy of the methylation procedure was determined by Fourier transform infrared (FTIR) spectroscopy, and indicated that approximately left half of the surface was methylated in water. Although, the treatments of the A549 cells were performed in culture medium instead of water, one can assume that the stability of ca. 50% of the methylation of the TiO_2 surface as monitored in water by IR is maintained in the buffered culture medium. In biological systems, the degree of hydrophilicity/hydrophobicity of a surface is considered to affect cell-surface adhesion, protein denaturation at the interface, and the selective adsorption of components from the liquid phase (Van Oss, 1994). Variations in the hydrophibicity of the surface can result in different translocation routes in various biological compartments, different coatings of the surface by endogenous materials, and differences in the interfacing of the solid with cells (Fubini et al., 1990). Specifically with regard to of TiO_2 the role of surface coating in its toxicity has been addressed in several *in vivo* investigations with contrasting outcomes (Oberdoster et al., 2001; Höhr et al., 2002; Rehn et al., 2002; Warheit et al., 2002).

Previously, we found some A mild reduction in pulmonary responses for methylated TiO_2 when compared to native counterpart was previously reported by some of us. However, the surface area per se appeared to be of much greater importance in causing contrasting responses (Höhr et al., 2002). Present investigations, allowed us to evaluate these *in vivo* observations in further detail in relation to cellular mechanisms.

In the present *in vitro* study, we observed a rapid uptake in A549 human lung epithelial cells for each of the TiO_2 samples, predominantly observed as membrane bound aggregates as well as large intracellular aggregates in vesicles, vacuoles and lamellar bodies. The internalisation of the TiO_2 particles in the A549 cells was confirmed by TEM/EDX and elemental mapping. Previously, Stearns *et al.* (2001) investigated the uptake of ultrafine TiO_2 in A549 cells. The particles used in their study had a primary particle size of about 50nm, which was roughly similar to the ultrafine sample used in our study. Although various experimental settings differed between both in the two studies (e.g. A549 culturing, particle sonication conditions, and treatment concentrations), over all findings were rather similar. In line with the findings by Stearns et al. (2001), particles appeared to be phagocytosed in agglomerates or clusters and no single ultrafine particles were was observed. Large aggregates of particles were also observed in association with the plasma membrane (within filopodia or lamellopodia) irrespective of their surface methylation. We also found TiO_2 particles (both fine and ultrafine) to be predominantly associated as large aggregates and/or their agglomerates, and these appeared to enter the cells by phagocytosis. These observations were well in line also with studies with fine and ultrafine TiO_2 in tracheal explants (Churg et al., 1998). For the ultrafine TiO_2, we occasionally observed endocytosis of small aggregates (<30nm) by clathrin coated vesicles, indicative of pinocytosis. Ingested particle aggregates were predominantly incorporated in membrane-bound vacuoles, and were also found associated with lamellar bodies, the main compartment of surfactant production. Multivesiculated bodies, which are regarded as acid phosphatase containing lysosomes mixed with residual membraneous material and other cell debris, were also found to contain TiO_2 particles. Other major organelles including the nucleus, mitochondria, Golgi apparatus and rough ER were all found to be devoid of TiO_2. As such, our present data are in contrast with observations by others, who found ambient ultrafine particles interacting within mitochondrial membranes (Li et al., 2003), or engineered SiO2 nanoparticles located inside the nucleus (Chen and von Mikecz, 2005). Our observations are also in contrast to recent investigations with spark-generated

ultrafine titanium particles, where single ultrafine particles were noted to locate inside cells as well as their nucleus (Geiser et al., 2005).

Semiquantitative analysis Inspection of the aggregate size distribution showed a tendency for the ultrafine TiO_2 particles to have aggregates in a lower size range (<200nm), when compared to the fine TiO_2 particles (200-500nm). Interestingly, for the fine particles, the methylation was associated with an apparent reduction of in the size of particle aggregated as observed within the A549 cells. This finding also provides some support that the surface methylation remained indeed effective under our cell experimental conditions. However, it can not be determined from our present analysis, to what extent the observed difference may have resulted from the increased hydrophilicity of this sample and possibly associated changes in the mechanisms or kinetics of phagocytosis. Notably, in this regard, the methylation effect on aggregation status was not observed for the ultrafine particles. The observed effect on aggregates may be due to the variation in hydrophilicity upon methylation. Note however that such effect is more pronounced on the fine than the ultrafine particles

In the present study, we also determined the The ability for of the different particle preparation to generate reactive oxygen species during treatment of the A549 cells is of paramount importance, since oxidative stress is considered to represent a hallmark of the toxic and inflammogenic effects of ultrafine particles (Donaldson et al., 2002; Oberdorster et al., 2005b). Therefore we chose EPR with the use of the Two spin-trapping agents, DMPO as well as and the spin-probe TEMPOL. DMPO, which is known to predominantly react with several free radicals by giving spectra having different hyperfine structure both ·OH and $O_2^{\cdot-}$, has been used by several investigators to determine generation of ROS in cell systems (Li *et al.*, 2000; Zhang *et al.*, 2001). TEMPOL is a low molecular weight, membrane permeable stable free radical with a well defined ESR spectrum. that is electron paramagnetic resonance detectable. TEMPOL is considered to act as a superoxide dismutate (SOD)-mimic in a redox mechanism involving oxidation by protonated superoxide to an oxoammonium cation, which in turn oxidises another superoxide to molecular oxygen. Hence, the The decay rate of the TEMPOL signal intensity, has been considered to reflect the production of superoxide, and has accordingly been used in cell-culture studies (Nagakawa *et al.*, 2000). Since the two spin-trapping agents show contrasting effects upon their reaction with ROS (i.e. an increase or reduction of the signal intensity for DMPO or TEMPOL respectively), false results due to possible artefacts (e.g. intracellular degradation, chemical degradation of the spin trap) are avoided. Interestingly, both with DMPO and TEMPOL we observed that the two different preparations of ultrafine TiO_2 elicited detectable increases in ROS generation during

treatment of the A549 cells, in contrast to their non-ultrafine counterparts. Furthermore, the effects as observed with the ultrafine TiO_2 were found to be irrespective of the methylation state, indicating that the increased surface area of the ultrafine particles per se, rather than their surface chemistry (i.e hydrophobicity), drives oxidative stress in the lung epithelial cells. Importantly, both spin-trapping agents show contrasting effects upon their reaction with ROS (i.e. an increase or reduction for DMPO or TEMPOL respectively), and thus the observed changes in EPR-signals appear not be due to possible artefacts such as intracellular degradation and or inactivation/binding with particles. Our findings in the cell cultures contrasted to the EPR results as obtained in the absence of cells. For DMPO, all samples showed increased DMPO-OH adduct levels above controls although these values did not reach statistical significance. However, with TEMPOL, no differences between any sample versus the controls were observed. Whether the The slight effects as observed with the DMPO were may be formation of artifacts, due to reactions with induction of free radical generation from light, the effect of particle sonication or metal impurities could not be verified. However, no differences were observed between the samples. Taken together, these data possibly indicates that the observed biological responses in our current study result from ROS as generated from the interactions of TiO_2 with the A549 cells, rather than as derived from the particle surface itself. However, it should be emphasised that the ESR settings as applied in our current study were specifically optimised for measurements under cell cultures. Specifically, DMPO and TEMPOL concentrations were used on the basis of their absence of cytotoxicity and stability on biological systems (Khan et al., 2003; May et al., 2005), and incubations were performed in particle suspensions placed in plastic dishes in an cell culture incubator. The radical generating properties of different TiO_2 samples in cell free environment are topic of ongoing investigations (Please add some lines and/or references here relating to your expertiese herein).

The ability of TiO_2 to generate ROS in acellular environment has been indicated from various independent studies, i.e. upon co-treatment with simulated sunlight/UV, with comparatively more pronounced effects for anatase than rutile (Wamer et al., 1997; Dunford et al., 1997; Hirakawa et al., 2004). In the absence of light irradiation, such effects are less conclusive, but tend to show stronger effects for ultrafine compared to fine TiO_2 (Donaldson et al., 1996; Dick et al., 2003). It should be noted that the ultrafine sample in our study contained 20% rutule, in econtrast to the fine fine) sample, which was pure anatase. It is not clear why the percentage of rutile/anatase should be relevant, specify.

It should be emphasised that both the ultrafine and the fine particles appeared as aggregates/agglomerates, yet they showed different responses. Although membrane bound NAPDH oxidases and/or mitochondria (Li et al., 2003; Knaapen et al., 2004) represent major candidates for these effects, we could not verify this as co-treatment of A549 cells with diphenyleneiodinium (DPI) yielded inconclusive results. Hence, further investigations are required to determine the mechanisms whereby interactions between ultrafine TiO_2 and lung epithelial cells lead to ROS generation. This will also be for importance in relation to toxicity screening as proposed for nanoparticles in general.

The effects of the different particle preparations on interleukin-8 expression in A549 cells were determined in this study for a number of reasons. Human lung epithelial cells are known to be an important sources of IL-8 in the lung, and this chemokine is well-recognised as an important mediator of pulmonary inflammation in humans (Kunkel et al., 1991; Keatings et al., 1996). Previous studies have shown that A549 cells are capable of expressing IL-8 upon exposure to various particles (Stringer et al., 1996; Simeonova and Luster, 1998; Schins et al., 2000). Most importantly, IL-8 is shown to be induced in A549 cells via a mechanism involving oxidative stress and acitvation of he redox-sensitive transcription factor Nuclear factor Kappa-B (Kunsch and Rosen, 1993; Stringer and Kobzik 1998; Schins and Donaldson, 2000). In the present study, we showed that both ultrafine samples, in contrast to their fine counterparts caused a significant release of interleukin-8 from the A549 cells, and this was asociated with their ability to enhance the IL-8 mRNA expression. DQ12 and TNFα were used as positive controls. The observed effects appeared to be independent of the methylation status of he samples. Importantly, endotoxin analysis of the samples showed that this effect was also not due to contamination of the particles with this well-known activator of IL-8 in A549 cells (Hansen et al 1997). Taken together, the effects on IL-8 release paralleled the effects on ROS generation as observed with the different TiO_2 samples. Notably, when compared on equal mass basis, a significant effect of DQ12 was observed at a five-fold lower concentration than for both TiO_2 sample preparations, irrespectively of the methylation status. These findings were in concordance with the five-fold difference in surface area for the fine (i.e. 10 m^2/g) versus the ultrafine (i.e. 50 m^2/g) samples. This aspect is further outlined in Figure 6, where IL-8 release from A549 cells is shown as a percentage above control as a fuction of the dose expressed as particle surface area per unit culture dish surface area (cm^2/cm^2). The graph indicates that, when considered at equal surface area, all four TiO_2 samples elicited a similar potency in causing IL-8 release. The figure also shows, that

compared to these samples, the quartz samples DQ12 has an intrinsically higher IL-8 inducing potency (See Figure 6). Current data also suggest that for particles with intrinsically low surface reactivity, such as TiO_2, the surface area per se is an important drive for inflammatory effects, and that the ultrafine TiO_2 samples used in this study are not intrinsically more active per unit surface area compared to the fine sample. Obviously, this may be different for other nanoparticles including those which are engineered and used specifically because of their "increased" reactivity on the nanoscale. The comparatively strong effect of the quartz sample is most likely due to its intrinsically higher surface reactivity (Fubini, 1997), and hence toxicity, when compared to relatively inert materials such as titanium dioxide in animal models (e.g. Driscoll et al., 1990; Duffin et al., 2002). At this stage, we would also like to emphasise that over all, high particle concentrations were required to observe any significant effect in the A549 cells: In view of its implications for risk assessment, these concentrations will extrapolate to estimated lung burdens that would require unrealistically high exposures to this material (Oberdorster and Yu, 1999). Nevertheless, present experiments allowed us to identify new insights on the potential mechanisms involved in the toxic effects of ultrafine (TiO_2) particles. Notably, in this regard our current in vitro observations were well in line with our previous *in vivo* investigations in rats with the same four TiO_2 samples. In both cases, relatively independent of the methylation status ultrafine TiO_2 showed stronger pulmonary responses after intratracheal instillation than fine TiO_2 (Höhr et al., 2002). Similarly, Rehn et al. (2002) compared the inflammatory effects of native (P25) versus silanised (P805) ultrafine titanium dioxide, but found only minimal, and not significantly differing inflammation, three days after a single instillation 0.15, 0.3, 0.6 and 1.2 mg. More interestingly, our present *in vitro* observations on the apparent similarities of all four TiO_2 samples in eliciting IL-8 release as a marker of inflammogenic potency upon considering dose in terms of applied surface area, is well in line with *in vivo* observations by other investgiators with TiO_2. As reviewed in Oberdorster et al. (2005b), instillation studies with two samples TiO_2, both in rat and mice, showed that neutrophilic inflammation was correlated to the total administered particle surface area, with the ultrafine and fine TiO2 fitting the same dose-response curve. Taken together with similar *in vivo* observations with other particles of different surface area (Tran et al., Inhal Toxicol; Duffin et al., 2002), our in vitro data provide further support that for relatively low toxicity particles of different sizes, such as TiO2, the administered total surface area is a better dosemetric than the administered mass or particle number (Oberdörster 2000).

In summary, both ultrafine TiO_2 samples but not their fine counterparts were found to elicit oxidative stress and interleukin-8 release from A549 cells, irrespective of their methylation, and despite the fact that these remained highly aggregated in cell culture as well as inside the cells. Our results indicate that ultrafine TiO_2, even as aggregates/agglomerates, has inflammatory properties that appear to be driven by their specific surface area. Furthermore, our results indicate that these effects are mediated by oxidative stress as elicited by particle-cell interactions, although the responsible mechanism(s) herein remain to be elucidated. Further research is also warranted to determined whether our current observations with TiO_2 are also applicable to other nanoparticles/ultrafine particles, including e.g. diesel exhaust particles which also typically exist as aggregates.

Acknowledgements

This study was supported by a grant from the German ministry of Environment (BMU) and partly by the Deutsche Forschungsgemeinschaft (DFG-Sonderforschungsbereich 503). We acknowledge Dr. Wolfgang Bischof (Institute of Occupational, Social and Environmental Medicine, University of Jena, Germany) for the endotoxin determinations and Chiara Sciolla (Department of Inorganic Chemistry, University of Torino, Italy) and Dr. Ad Knaapen (Maastricht University, The Netherlands) for their helpful contributions to the initial experiments related to this work.

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FIGURES

Figure 1. Fourier-Transformed Infrared spectra of ultrafine titanium dioxide before and after methylation. The IR spectra reveals the absorbance bands which are attributed to the presence of distinctive CH_3 and H_2O groups on the particles. The graph represents UF-TiO_2 after outgassing at room temperature (spectrum a), after treatment at 250°C in CH_3OH (40 torr) for 45 minutes with subsequent outgassing (spectrum b), and after readmission of H_2O at room temperature(spectrum c).

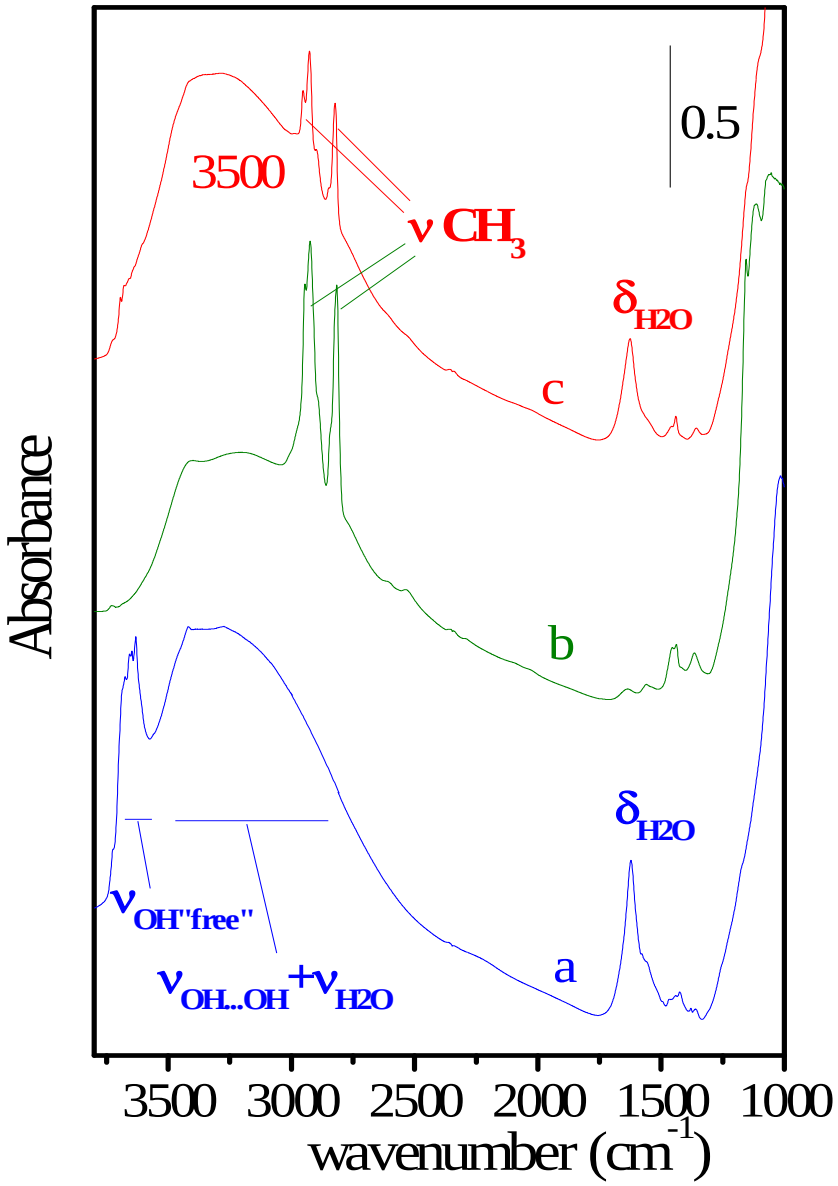


Figure 2. Transmission electron micrographs of endocytosis of titanium dioxide particles by A549 cells. Representative pictures are shown for A549 cells upon treatment with 16µg/cm^2 ultrafine TiO_2 for 4hrs. Upper left panel: lamellipodia engulfing TiO_2 particle aggregates. Lower left panel: localisation of particles inside a lamellar body. Right panels: membrane bound aggregates of particles near mitochondria (upper right panel) and nuclear membrane (lower right panel).

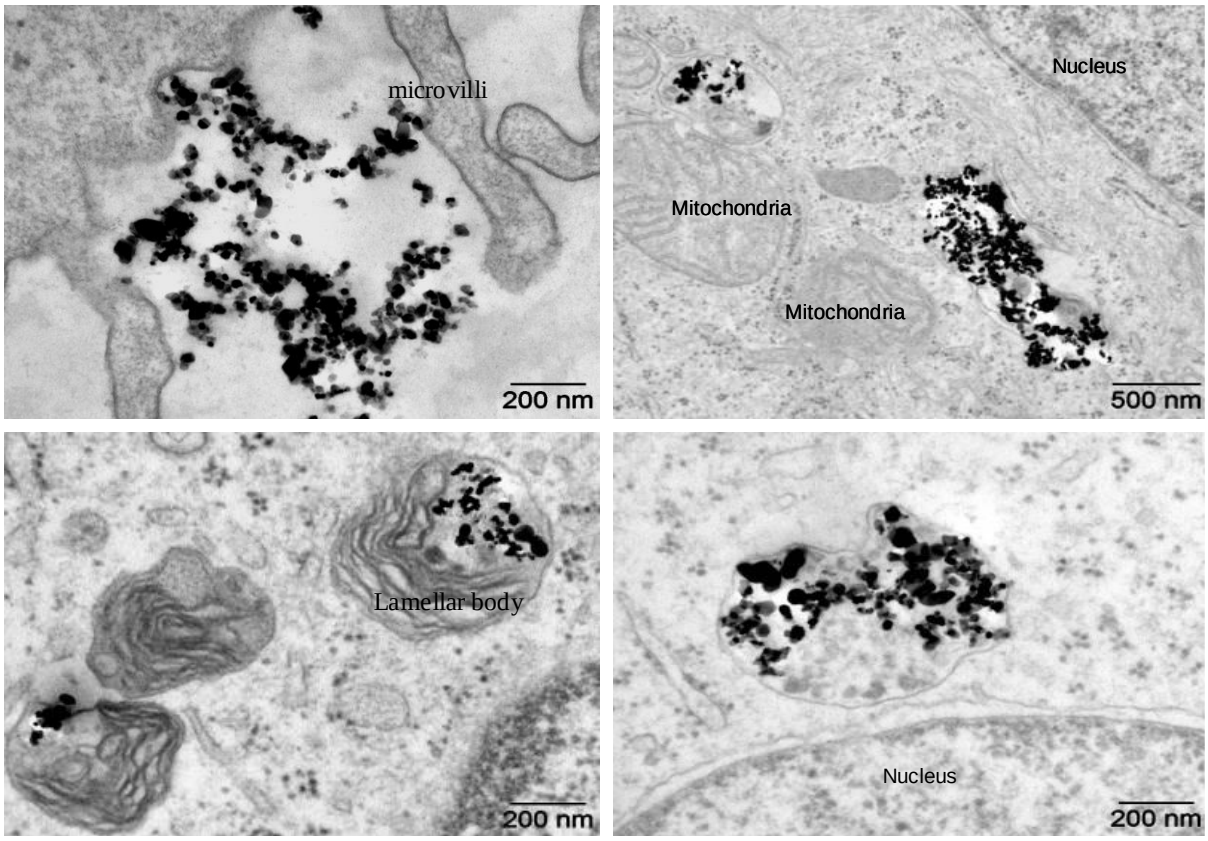


Figure 3. Comparison of aggregate size distribution (nm) for the four different preparations of TiO_2 following uptake in A549 cells. Each sample was incubated for 4 hours at a dose of 16 μg/cm^2. Aggregate diameters in A549 cells were measured using transmission electron microscopy with software assisted analysis.

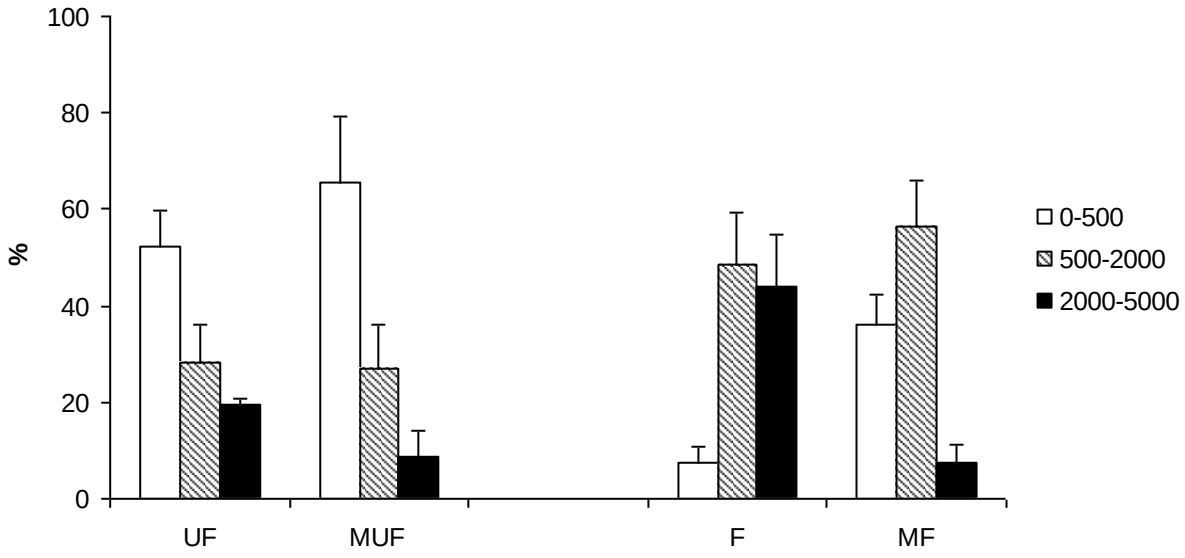


Figure 4. Reactive oxygen species formation from A549 cells upon treatment with the different dust preparations. The different TiO_2 samples were suspended in HBSS, sonicated and then immediately added to the A549 cells for a total of 2 or 4 hours in HBSS at 400µg/cm^2. One our after the start of the treatment DMPO or TEMPOL were added. At the end of each incubation, the medium was aspired and analysed with ESR. Data represents the mean and standard errors of three independent experiments (ANOVA with Dunnett post hoc comparison). Panel A: Results with DMPO. Panel B: Results with TEMPOL. P<0.05 * and P<0.01** versus control (=C). F=fine, MF=methylated fine; UF=ultrafine; MUF=methylated ultrafine. The inserts show the signals as observed after 4h in the absence of A549 cells.

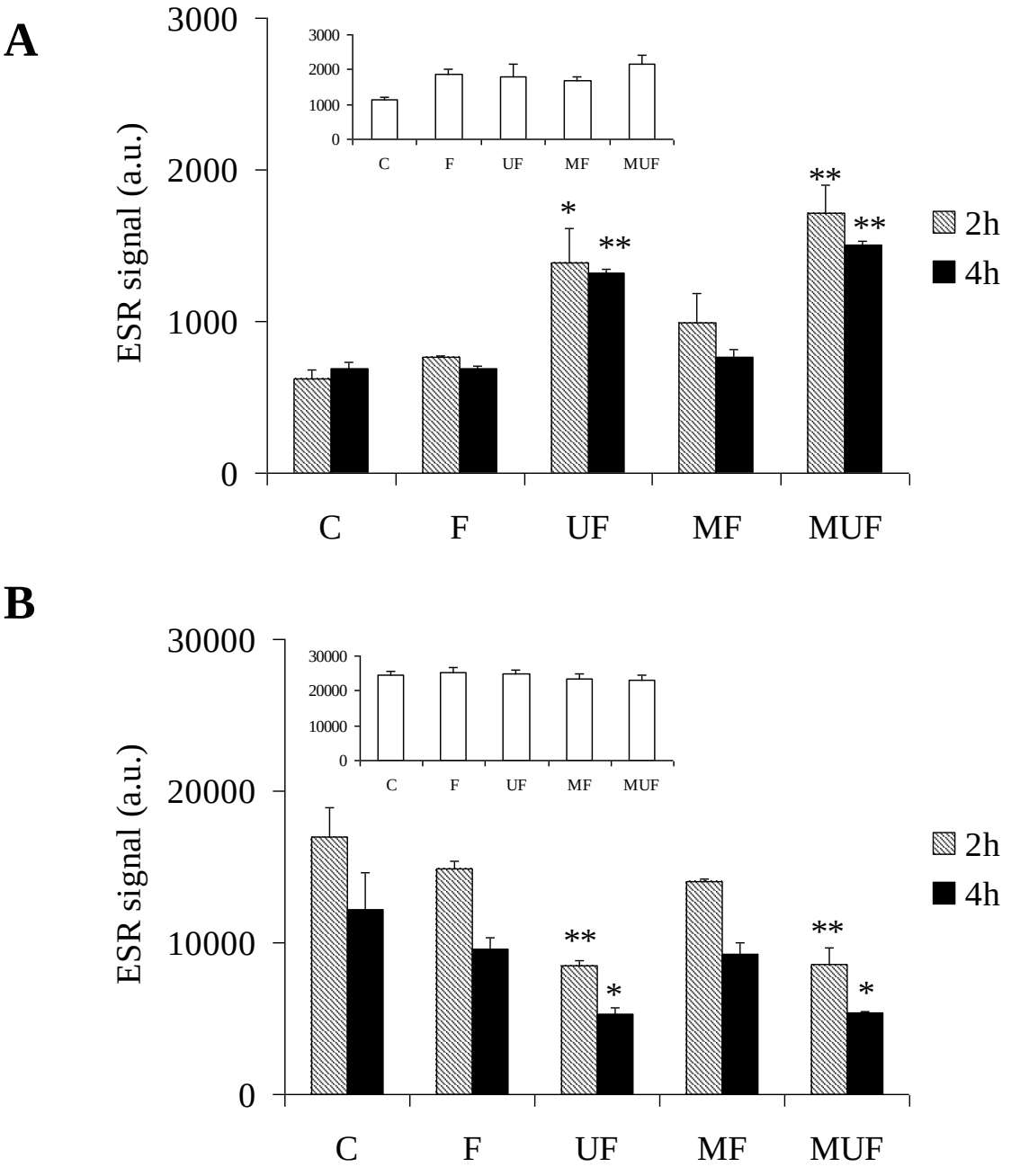
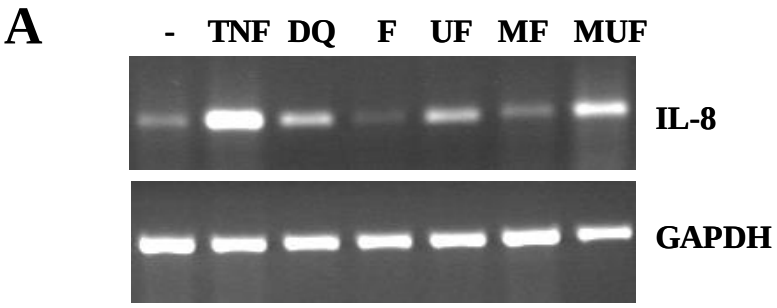


Figure 6. IL-8 mRNA expression and protein release from A549 cells upon treatment with the different preparations of titanium dioxide. Panel A: Representative gel showing interleukin-8 mRNA expression in A549 cells upon 4h treatment with the different tianium dioxide samples (80µg/cm^2). TNF-alpha (10ng/ml) or DQ12 quartz (80µg/cm^2) were used as positive controls, and GAPDH was used as housekeeping gene. Tumor Necrosis Factor-alpha (TNF) and quartz particles (DQ12) were used as positive controls. Panel B: Release of IL-8 after 24h treatment of cells A549 with the different titanium dioxide particles. F=fine, MF=methylated fine; UF=ultrafine; MUF=methylated ultrafine. DQ = Quartz particles (DQ12) were used as positive control. Data are mean and standard errors of n=3 independent experiments (*p<0.05 vs. untreated cells).



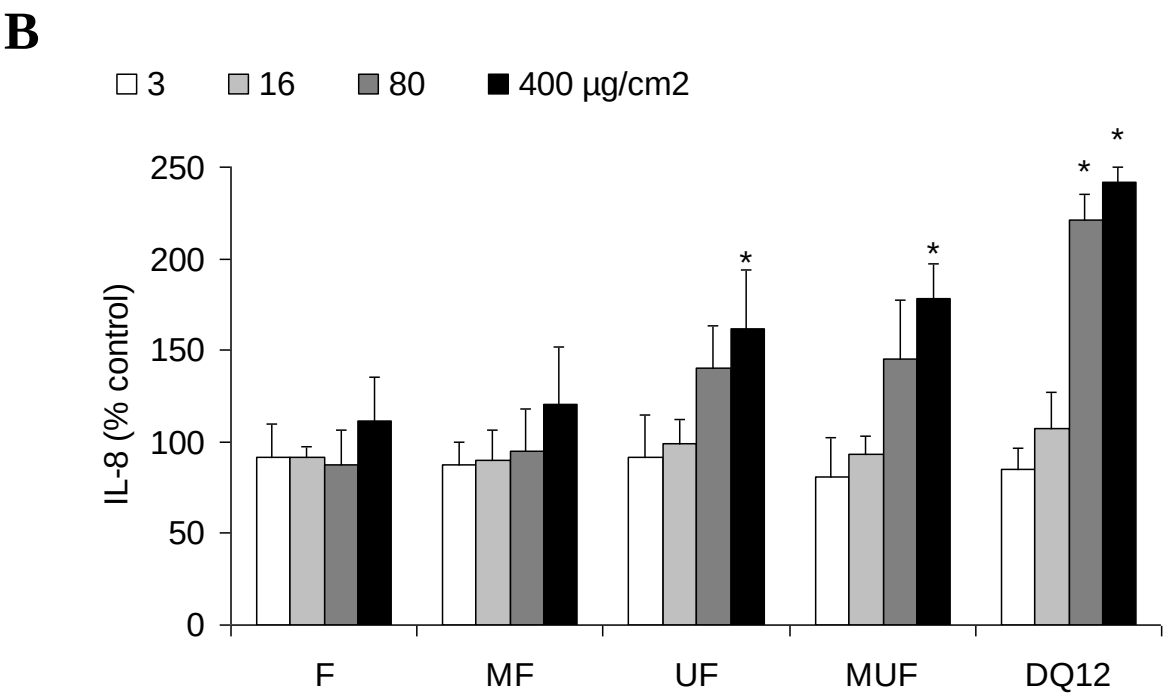


Figure 7. Release of interleukin-8 from A549 cells as function of particle surface dose. Interleukin-8 expression is shown as a perecentage above control as fuction of the dose expressed as particle surface area per A549 monolayer surface area (cm^2/cm^2). The graph indicates that when considered at equal surface area, all TiO_2 samples elicited similar potency in causing IL-8 release, whereas DQ12 has intrinsically higher IL-8 inducing potency.