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In vitro investigation of oxide nanoparticle and carbon nanotube toxicity and intracellular accumulation in A549 human pneumocytes

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ABSTRACT

If released in the environment, nanomaterials might be inhaled by populations and cause damage to the deepest regions of the respiratory tract, i.e., the alveolar compartment. To model this situation, we studied the response of A549 human pneumocytes after exposure to aluminium oxide or titanium oxide nanoparticles, and to multi-walled carbon nanotubes. The influence of size, crystalline structure and chemical composition was investigated. After a detailed identification of nanomaterial physico-chemical characteristics, cells were exposed *in vitro* and viability and intracellular accumulation were assessed. In our conditions, carbon nanotubes were more toxic than metal oxide nanoparticles. Our results confirmed that both nanotubes and nanoparticles are able to rapidly enter into cells, and distribute in the cytoplasm and intracellular vesicles. Among nanoparticles, we demonstrate significant difference in biological response as a function of size, crystalline phase and chemical composition. Their toxicity was globally lower than nanotubes toxicity. Among nanotubes, the length did not influence cytotoxicity, neither the presence of metal catalyst impurities.

1. Introduction

The massive development of nanotechnologies leads to considerable concern regarding the potential biological effects and human toxicity of nanomaterials (Oberdorster et al., 2005a,b). The lung, which is directly exposed after inhalation of contaminated air, is one of the first target organs of nanomaterials. Due to their size, nanomaterials distribute in the whole respiratory tract and can reach pulmonary alveoli (Borm et al., 2006; Nel et al., 2006; Oberdorster et al., 2005a,b).

In vitro biological impact on lung of titanium oxide nanoparticles (NP-TiO_2) was addressed on bronchial epithelial cells (Gurr et al., 2005), lung macrophages (Soto et al., 2007) and with the A549 human type II alveolar epithelium cell line (Park et al., 2007; Sayes et al., 2006; Singh et al., 2007; Soto et al., 2007; Stearns et al., 2001). The cytotoxicity of NP-TiO_2 depends on the particles morphology and crystalline phase. Sayes et al. (2006) demonstrate a classic dose–response and time-dependent cytotoxicity of NP-TiO_2 at high concentrations (>100 μg/ml), anatase NP-TiO_2 being more cytotoxic than rutile NP-TiO_2 of the same size (5–10 nm, loosely agglomerated): in their study cytotoxicity thus does not depend on nanoparticles size but rather on phase composition. Park et al. (2007) observe cytotoxicity of 30 nm nanoparticles and 1 μm particles of TiO_2 at concentrations higher than 50 μg/ml, the largest particles leading to the most important morphological changes in cells. On the contrary, Soto et al. (2007) observe no cytotoxicity of 5–40 nm anatase and 2–60 nm rutile TiO_2 nanoparticles on murine and human alveolar macrophages, and a low toxicity of these nanoparticles on A549 cells.

In vitro, the biological impact of multi-walled carbon nanotubes (MWCNT) was investigated on various respiratory models (Jia et al., 2005; Magrez et al., 2006; Muller et al., 2005; Pulskamp et al., 2007). Multiple processes of synthesis, leading to various types of MWCNTs render difficult the comparison of their biological effects. As a function of the considered publication, parameters influencing their toxicity include surface structure (Bottini et al., 2006; Magrez et al., 2006; Tian et al., 2006); length (Sato et al., 2005); agglomeration state, i.e., presence of a dispersing agent; and contamination of metal catalyst and amorphous carbon (Pulskamp et al., 2007). For example, on A549 cells, it is the presence of metal trace impurities contained in MWCNT which causes the release of reactive oxygen species (ROS) and the decrease of mitochondrial membrane potential (Pulskamp et al., 2007).

Results from these studies still need to be validated. They are often controversial and every condition leads to a different biological response. Still no correlation can be identified between the

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biological impact of nanomaterials and their size, surface structure, crystalline phase, chemical composition, presence of metal trace impurities. Moreover nanomaterials are not always fully characterized before and in the course of the experiment. Finally, to our knowledge, very few studies directly compared the impact of metal oxide nanoparticles and carbon nanotubes on the same cell model, during the same experiment.

Our aim was to compare the biological response of A549 cells to nanomaterials of different forms (particles or tubes), size, crystalline phase and chemical composition. Here we report the first results of our investigations: cytotoxicity effects and accumulation. The originality of the study is that it was done with a panel of well-characterized nanomaterials on the same cell model. Metal oxide nanoparticles (TiO_2 and Al_2O_3) and multi-walled carbon nanotubes were chosen since they represent, today, the most produced nanomaterials, and are already integrated in the composition of domestic goods (http://www.nanotechproject.org). TiO_2 nanoparticles are either rutile or anatase, their size range from 12 to 140 nm. Al_2O_3 diameter is comparable to one of the TiO_2 nanoparticles under study. The carbon nanotubes considered here differ in terms of length and presence of catalyst impurities and are well dispersed. For their purification we did not use any acid treatment to avoid the formation of highly reactive sites at the surface of the nanotubes. After a detailed characterization of these nanomaterials, cytotoxicity and intracellular accumulation were taken as endpoints. Three-independent cytotoxicity assays were used to avoid the artefacts involved by adsorptive properties of nanotubes (Casey et al., 2007b; Worle-Knirsch et al., 2007). Intracellular accumulation of nanomaterials was observed, together with morphological damage in cells, by transmission electron microscopy (TEM).

2. Materials and methods

2.1. Chemicals

Chemicals and cell culture media and supplement were obtained from Sigma–Aldrich (Saint-Quentin Fallavier, France).

2.2. Nanomaterials

2.2.1. Powders

MWCNT and TiO_2-CEA originate from our institute (Francis Perrin Laboratory, CEA Saclay, France). MWCNT were synthesized by aerosol-assisted catalytic chemical vapour deposition (CVD) using Fe as catalyst (Pinault et al., 2005). Raw MWCNT are 500 μm long and well aligned. They contain residual iron-based catalyst mainly encapsulated in the central core of MWCNT and almost no by-product such as amorphous carbon and encapsulated metallic particles. After synthesis, nanotubes were heated under argon atmosphere at 2000 °C during 2 h in order to remove iron-based phases (Pinault et al., 2005). Therefore, iron content in raw MWCNT and in annealed MWCNT is 4.24 and 0.08 wt.%, respectively. TiO_2-CEA nanoparticles were synthesized by laser pyrolysis (Pignon et al., 2008), and then annealed under air at 400 °C during 3 h to remove free carbon impurities. DRX analysis determined that their crystalline structure is 95% anatase. The effects of these nanotubes and nanoparticles were compared to effects of commercial nanoparticles. TiO_2-P25 (Aeroxide® P25, 75% anatase) and Al_2O_3 (Aeroxide® AluC) nanoparticles were obtained from Degussa AG (Düsseldorf, Germany). TiO_2-Sigma-rutile (ref. 637262, 100% rutile) and TiO_2-Sigma (ref. T8141, 100% anatase) were obtained from Sigma–Aldrich (Saint-Quentin Fallavier, France).

2.2.2. Suspensions

Suspensions of nanomaterials were prepared in ultrapure sterile water (pH 5.5). For nanotubes, Arabic gum (0.25 wt.%) was added to sterile water. Nanomaterials were dispersed by sonication (Autotune 750 W, Bioblock Scientific) at 4 °C, using a pulsed mode, during 30 min for nanoparticles, 30 min for nanotubes having the longest size (designated "long nanotubes") and 8 h for the shorter ones (designated "short nanotubes"). Indeed the sonication is known to induce breakage of the nanotubes, the final length of the nanotube samples will be shorter for the longer duration (Glory et al., 2007). The concentration of suspensions was 1 g/L for nanotubes and 10 g/L for nanoparticles. These suspensions will be called "stock suspensions" in the following text.

For cell exposure, stock suspensions were diluted in cell culture medium (DMEM medium supplemented with 50 UI/ml penicillin and 50 μg/ml streptomycin). These suspensions will be called "exposure suspensions".

2.3. Characterization of nanomaterial suspensions

The characteristic of nanoparticle powders are summarized in Table 1. The morphologies and sizes of nanomaterials in stock suspensions were determined by transmission electron microscopy (TEM), using a Philips EM208 microscope at 80 kV (CCME Orsay, France). A drop of stock suspension was deposited on a TEM grid, allowed to dry and directly observed. Size was measured on 150–200 nanoparticles or nanotubes, randomly chosen. Specific surface areas were measured by the classical method of Brunauer Emmett and Teller (BET) consisting in isothermal gas-adsorption (Brunauer et al., 1938) using a Micromeritics Flowsorb 2300 (Norcross, USA). For round-shaped nanoparticles, BET diameter was calculated as $D = 6/(\rho \times SSA)$, where D (m) is BET diameter, ρ (g/cm^3) is the density of nanomaterial and SSA (m^2/g) is the specific surface area. Density of nanomaterials was taken as $\rho = 3.90$ for anatase TiO_2 and 3.99 for Al_2O_3.

In exposure suspensions, agglomeration state was identified by photon correlation spectroscopy (PCS). This technique cannot be applied to non-spherical nanomaterials, such as nanotubes and rutile nanoparticles. Isoelectric point was determined by zeta potential measurement. Both parameters were determined using a Malvern Zetasizer 3000HS (Malvern, Worcestershire, UK) equipped with MPT-1 titrator.

2.4. Cell culture

A549 human type II lung epithelium cell line, classically used for lung toxicity assays, was obtained from American Type Culture Collection (ATCC, USA, CCL-185) (Lieber et al., 1976). They were grown in DMEM supplemented with 10% (v/v) fetal bovine serum, 2 mM L-glutamine, 50 UI/ml penicillin and 50 μg/ml streptomycin. They were maintained at 37 °C in a 5% CO_2 and passed at 80% of confluence. Exposures to nanomaterials were done on sub-confluent cells.

2.5. Toxicity assays

Cells were seeded in 96-well plates. After 24 h they were exposed to 100 μL of exposure suspensions of nanomaterials, from 0.25 to 100 μg/ml during 1 to 72 h (see figure legends for details). Fetal bovine serum was avoided since it was proven to interact with nanotubes (Casey et al., 2007b).

Interactions of single-walled carbon nanotubes with several dyes commonly used for cytotoxicity testing were demonstrated (Casey et al., 2007a; Worle-Knirsch et al., 2007). This interaction has never been demonstrated for MWCNT. Among these tests, LDH, WST-1 and XTT assays are considered reliable. Consequently, we performed a battery of classical cytotoxicity tests in order to ensure that our results were not false positive, and in order to detect any interaction between MWCNT and cytotoxicity assays. Cell metabolic activity, reflecting nanomaterial cytotoxicity, was assessed by using 3-(4,5-dimethylthiazol-z-yl)-2,5-dipheny-tetrazotium bromide

Table 1
Characteristics of nanomaterials[a].

	Supplier, reference	Morphology	Size TEM (measured)	SSA (m^2/g)	Size BET (calculated)	Ip
Al_2O_3	Degussa, Aeroxide® AluC	Spherical	11 ± 3 nm (13 nm)	83 ± 1 (100 ± 15)	18	8.4
TiO_2-Degussa-P25	Degussa, Aeroxide® TiO_2 P25	Spherical	25 ± 7 nm (21 nm)	46 ± 1 (50 ± 15)	33	7
TiO_2-CEA	CEA	Spherical	12 ± 3 nm	82 ± 4	19	6.4
TiO_2-Sigma	Sigma, T8141	Spherical	142 ± 36 nm (<44 μm)	10 ± 1	152	
TiO_2-Sigma-rutile	Sigma, 637262	Elongated	*L*: 68 ± 17 nm (<100 nm), *D*: 9 ± 3 nm	118 ± 1	–	
Long nanotubes	CEA	Elongated	*L*[0.1–12] μm, *D*[10–160] nm	42 ± 2	–	–
Short nanotubes	CEA	Elongated	*L*[0.1–3.5] μm, *D*[10–160] nm	42 ± 2	–	–

[a] Size was determined by transmission electron microscopy observation. SSA: specific surface area, measured according to Brunauer Emmett and Teller protocol. The indication into brackets is the theoretical values given by the supplier. Ip: isoelectric point obtained by zeta potential measurements. Crystal structure was deduced from XRD diagram. *L*: length, *D*: diameter.