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To cite this article: Sabrina Barillet et al 2009 J. Phys.: Conf. Ser. 170 012016

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SiC nanoparticles cyto- and genotoxicity to Hep-G2 cells

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Abstract. While emerging nanotechnologies have seen significant development in recent years, knowledge on exposure levels as well as data on toxicity of nanoparticles are still quite limited. Indeed, there is a general agreement that development of nanotechnologies may lead to considerable dissemination of nanoparticles in the environment. Nevertheless, questions relative to toxicity versus innocuousness of such materials still remain. Our present study has thus been carried out with the purpose of assessing some aspects of toxicological capacities of three kinds of nano-sized particles: TiO₂ and SiC nanoparticles, as well as multi-walled carbon nanotubes (CNT). In order to address the question of their potential toxicity toward living cells, we chose several cellular models. Assuming inhalation as the most probable exposure scenario, we used A549 alveolar epithelial cells as a model for mammalian primary target organ (lung). Furthermore, we considered that nanoparticles that would deposit into the pulmonary system may be translocated to the circulatory system. Thus, we decided to study the effect of nanoparticles on potentially secondary target organs: liver (WIF-B9, Can-10, HepG2) and kidneys (NRK-52E, LLC-PK1). Herein, we will focus our attention on results obtained on the HepG2 cell line exposed to SiC nanoparticles. Scarce literature exists on SiC nanotoxicology. According to the authors that have already carried out studies on this particular nanoparticle, it would seem that SiC nanoparticles do not induce cytotoxicity. That is one of the reasons of the potential use of these nanoparticles as biological labels [1]. We thus were interested in acquiring more data on biological effects induced by SiC nanoparticles. Furthermore, one of the particular aspects of the present study lies in the fact that we tried to specify the influence of physico-chemical characteristics of nanoparticles on toxicological endpoints (cytotoxicity and genotoxicity).

1. Materials and methods

1.1. Nanoparticle suspensions
SiC nanopowders were synthesized by laser pyrolysis [2, 3]. Their specific surface area was measured according to Brunauer, Emmet and Teller (BET) method [4]. Diameter of powdered nanoparticles could be calculated from BET results. Nanoparticles were first dispersed by sonication (Autotune 750 W, Bioblock Scientific) in ultrapure sterile water (4°C, pulsed mode: 1 s on and 1 s off, 30 min),
then twice-diluted in foetal bovine serum, before being sonicated another time (same protocol). Morphology was determined by transmission electron microscopy (TEM), using a Philips EM208 microscope at 80 kV (CCME Orsay, France): a drop of each nanoparticle suspension was deposited on a TEM grid, allowed to dry and directly observed. For cells exposure, these suspensions were diluted ten times in cell culture medium (DMEM medium supplemented with 50 IU/mL penicillin and 50 µg/mL streptomycin).

1.2. Cell culture
HepG2 cells (human Hepatocellular carcinoma) were purchased from ATCC and subcultured in DMEM containing 4.5 g/L glucose supplemented with 2 mM L-glutamine, penicillin/streptomycin (50 IU/mL and 50 µg/mL, respectively) and 10% (v/v) fetal bovine. They were maintained at 37°C in a 5% CO₂/air incubator and passed at confluence.

1.3. Cytotoxicity assay
Nanoparticles cytotoxicity was assessed by using 3-(4,5-dimethylthiazol-z-yl)-2,5-dipheny-tetrazonium bromide (MTT) assay [5]. Mitochondrial dehydrogenases of viable cells reduce MTT to water-insoluble blue formazan crystals which are then solubilised by dimethyl sulfoxide (DMSO); this assay thus indicates cell mitochondrial activity impairment. After exposure, 10 µL of a 5 mg/mL MTT solution were added to each well. After 1 h at 37°C, medium was then replaced by 100 µL of DMSO and mixed thoroughly to dissolve the formazan crystals. Nanoparticles were allowed to sediment during 1 h and 50 µL of each well were then transferred to another plate. Absorbance was measured at 570 nm. Cell viability was determined as a percentage of the negative control (untreated cells).

1.4. Genotoxicity assay
Comet assay was performed under alkaline conditions. Cells were embedded in low melting point agarose (LMPA) before being deposited on precoated microscope slides and covered by a coverslip. The slides were placed on a flat tray and kept on ice for 5 min until agarose solidifies. The coverslips were removed and another layer of 0.5% LMPA was dispensed, covered by a coverslip and allowed to solidify on ice. The slides were immersed in a cold lysing solution (2.5 M NaCl, 100 mM EDTA, 10 mM Tris, 10% DMSO, 1% Triton X-100) for 1 h. DNA was then allowed to unwind for 40 min in an alkaline electrophoresis solution (300 mM NaOH, 1 mM EDTA, pH>13). Electrophoresis was performed in a field of 0.7 V/cm and 300 mA current for 24 min. Slides were neutralized with 0.4 M Tris pH7.5 and stained with 50 µL of 20 µg/mL ethidium bromide. At least 50 comets per sample were analyzed under a fluorescence microscope (Olympus BX41) equipped with a 350-390 nm excitation and 456 nm emission filter at x20 magnification. Comet parameters were analyzed by using Comet IV software (Perceptive Instruments, Suffolk, UK).

1.5. Statistical analysis
Results are expressed as mean ± standard deviation of at least three assays. Statistical evaluation was performed with STATISTICA software (Statsoft). One-way Kruskal-Wallis ANOVA was used, followed by Mann-Whitney U-tests. p<0.05 was considered to be statistically significant.

2. Results and discussion
2.1. Physico-chemical characterization
All these nanoparticles were spherical. A TEM picture is presented in Figure 1. After the first sonication step, in water, nanoparticles were still agglomerated into large clusters of 800-2000 nm. After the second step of sonication, suspension dispersion state was better, but nanoparticles were still agglomerated into 50-100 nm clusters. These suspensions were stable for almost 1 month.
Table 1. Nanoparticles characteristics.

| Dimensions | Size BET (nm) | SSA (m²/g) | Si/C ratio |
|------------|---------------|------------|------------|
| 1          | 19.95         | 94         | 1.27       |
| 2          | 15.00         | 125        | 0.83       |
| 3          | 13.99         | 134        | 1.01       |
| 4          | 13.39         | 140        | 1.24       |
| 5          | 36.06         | 52         | 1.14       |
| 6          | 57.52         | 33         | 1.13       |

SSA: Specific surface area, measured according to Brunauer Emmett and Teller protocol.

Figure 1. Transmission electron microscopy image of a nanoparticle suspension (SiC#3). Nanopowder was first dispersed by sonication (Autotune 750 W, Bioblock Scientific) in ultrapure sterile water (4°C, pulsed mode: 1 s on and 1 s off, 30 min), then twice-diluted in foetal bovine serum, before being sonicated another time (same protocol). A drop of suspension was then deposited on a TEM grid, allowed to dry and directly observed by TEM, using a Philips EM208 microscope at 80 kV (CCME Orsay, France).

2.2. Cytotoxicity

Results relative to cytotoxicity assay are presented in Figure 2.

From a general point of view, we observed that, whatever the nanoparticle type (SiC#1, #2, #3, #4, #5 or #6), concentration (from 1 to 200 µg/mL), or time of exposure (24 or 48 h), mortality never affected more than a third of the total population. Indeed, the most drastic conditions (200 µg/mL during 48 h) only led to about 30% of mortality with SiC#1, #2, #4 and #5 nanoparticles, or to about 20% or mortality with SiC #3 and #6 (the latter one inducing the least cytotoxic effects with only 17% of cell death). This first consideration tends to demonstrate that cytotoxic effects of SiC nanoparticles are quite limited.

Considering the effects of time exposure on nanoparticles cytotoxicity, we could notice that, for some nanoparticles, major parts of the cytotoxic effects occurred during the first 24 h of exposure. The most obvious example of that trend can be seen considering results obtained after SiC#5 exposure. In this case, curves obtained after 24 and 48 h of exposure are almost perfectly superimposed. This trend was also noticed for SiC#4 (dead cells making up 19% and 29% of the total population after 24 h and 48 h of exposure respectively). Conversely, SiC#3 nanoparticle seemed to induce very weak cytotoxic effects after the first 24 h of exposure (5% cell death at 200 µg/mL nanoparticles) against more marked effects at the end of a 48 h exposure period (21% at the same concentration).

As regard the nanoparticles concentration effect on cytotoxicity, we could note that cell death is globally concentration-dependant. For some nanoparticles (#1, #2, #5), this relationship remained
throughout the tested concentrations panel (from 5 to 200 µg/mL). For others nanoparticles (#3, #4, #6), cytotoxic effects seemed to reach a plateau at the highest concentrations. Finally, nanoparticles physico-chemical characteristics (size, chemical composition) did not seem to significantly affect their impact on cell viability.

![Figure 2. Nanoparticles cytotoxicity. HepG2 cells were exposed 24 h (dashed line) or 48 h (solid line) to nanoparticles and cell mortality was assessed by MTT assay (asterisks denote statistical significance, p<0.05).](image)

2.3. Genotoxicity
Previous studies carried out in our laboratory tended to establish a significant relationship between nanoparticles exposure and intracellular oxidative stress (intracytoplasmic ROS overproduction, significant changes in antioxidant enzyme activities). Thus, we decided to investigate possible induction of DNA damage. However, first experimental results seem to invalidate the hypothesis of a genotoxic injury induced by SiC nanoparticles.

3. Conclusion
In this study we show that silicon carbide nanoparticles induced a slight but significant cell death. Nevertheless, it has to be noted that nanoparticles concentrations required to observe such impact are very important, and could not be appreciated as environmentally realistic. However, in case of a specific organotropism toward organs and tissues such as liver or kidney, nanoparticle concentrations could be locally disproportionately elevated and such cell response would potentially be observed. DNA damage could not be established as a consequence of SiC nanoparticles exposure.
Considering the lack of knowledge as regards nanotoxicology, these first experimental results can be considered as a starting point for SiC nanotoxicological studies. Obviously, more complex mechanisms than just genotoxic injury need to be investigated in order to have a clearer view on nanoparticles modes of action.

Acknowledgement
This work was supported by ADEME (Agence de l’Environnement et de la Maîtrise de l’Énergie), by the Région Ile-de-France in the framework of C’nano IdF. C’Nano-IdF is the nanoscience competence center of Paris Region, supported by CNRS, CEA, MESR and Région Ile-de-France. It was also funded by the French National Research Agency (ANR).

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