In vitro cellular responses to silicon carbide nanoparticles: impact of physico-chemical features on pro-inflammatory and pro-oxidative effects

UNIVERSITÀ DEGLI STUDI DI TORINO

In vitro cellular responses to industrial silicon carbide particles: impact of physico-chemical features on pro-inflammatory and pro-oxidative effects

Delphine Boudard* †∂§, Valérie Forest ∂‡, Jérémie Pourchez ∂‡, Najih Boumahdi ∂Ψ, Maura Tomatis ♇, Bice Fubini ♇, Bernard Guilhot ∂Ψ, Michèle Cottier †∂§, Philippe Grosseau ∂Ψ

† Université Jean Monnet Saint-Etienne, F-42023, Saint-Etienne, France, EA 4624 LINA

∂ SFR IFRESIS, F-42023, Saint-Etienne, France

§ CHU de Saint-Etienne, F-42055, Saint-Etienne, France

‡ Ecole Nationale Supérieure des Mines, CIS-EMSE, EA 4624 LINA, F-42023 Saint-Etienne, France

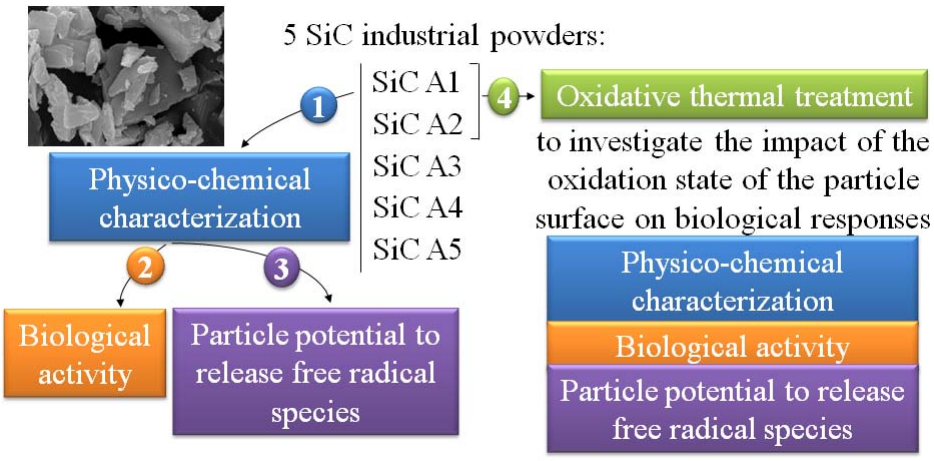
Ψ Ecole Nationale Supérieure des Mines, SPIN-EMSE, CNRS: UMR5307, LGF, F-42023 Saint-Etienne, France

♇ Dipartimento di Chimica and 'G. Scansetti' Interdepartmental Center for Studies on Asbestos and other Toxic Particulates, Università di Torino, Torino, Italy

*Correspondence to: Delphine Boudard, EA 4624 LINA, Université Jean Monnet, Faculté de Médecine, 15 rue Ambroise Paré, F-42023, Saint-Etienne, France. Phone : (+33) 04 77 42 14 43, FAX : (+33) 04 77 42 14 89, E-mail : delphine.boudard@univ-st-etienne.fr

***Keywords**: silicon carbide powders, Acheson process, thermal post-treatment, physico-chemical characterization, biological toxicity, occupational exposure.*

Table of content



Abstract

Silicon carbide (SiC), largely employed in various industrial applications is exclusively produced via the Acheson process. SiC toxicity has been poorly investigated. Our study aims at characterizing the physico-chemical features of 5 SiC powders manufactured in different sizes (designed SiC A1 to SiC A5) which could have a direct impact on their biological activity. Macrophages from the RAW 264.7 cell line were exposed to the different SiC particles and the *in vitro* responses were evaluated. Opposite to what happens with silicas no cytotoxicity was observed (no membrane damage as assessed by LDH release) but pro-oxidative reactions (H_2O_2 production) and pro-inflammatory responses of variable intensity were evidenced. Oxidative stress appeared related to particle size (fine versus coarse particles), while the iron level regulated inflammogenicity as evaluated through TNF-α production. To investigate the impact of surface reactivity on the biological responses, coarse SiC A1 and fine SiC A2 powders were submitted to different thermal treatments (650, 750, 850 and 1400°C) in order to alter the oxidation state of the particle surface. A strong correlation was observed between size and biological activity especially oxidative stress. At 1400°C a decrease in TNF-α production and an increase in $HO^{\bullet}$, $COO^{\bullet -}$ radical production were observed in correlation with the formation of a surface layer of crystalline silica.

Introduction

Silicon carbide (SiC) was discovered in 1892 by Edward Goodrich Acheson who searched a diamond substitute material[1]. Today SiC denomination is a generic term that refers to a family of different materials sharing some common physico-chemical characteristics. The production of SiC, a well known material, has significantly increased during the past fifty years mainly due to its remarkable properties such as high chemical inertness, elevated thermal stability and excellent mechanical properties. SiC particles are widely used for many different industrial purposes in the ceramic or composite material field, including particulate filters and abrasives[2].

SiC is industrially synthesized by the Acheson process from reactants (petroleum coke and silica), placed all around a graphite resistance in an electric furnace, to enable carbothermal reduction of SiO_2 into SiC at high temperature. At the end of the firing and after several post-process operations (crushing, grinding, etc.) polydisperse SiC powders are obtained. At each step of this process, aerosolized SiC fractions as well as by-products, can be generated in the work environment and potentially inhaled by the workers. A health risk among SiC workers has been noticed since the beginning of the 19th century[3, 4]. Occupational analyses showed that the airborne environment was charged with SiC microparticles, SiC fibers and impurities, especially crystalline silica and carbon[5, 6]. Moreover, analyses of lung tissue biopsies from workers exposed to these powders have revealed substantial amounts of SiC particles in alveolar spaces and interstitial fibrosis associated with an increased pneumoconiosis incidence[7, 5, 8, 9]. Taken together, these observations were considered to be the result of a silicogenic effect and showed the importance of occupational risk evaluation on SiC exposure, through preventive epidemiological and toxicological studies[10–12].

Toxicological data available in the literature are mainly obtained from experiments conducted on micro-scaled SiC particles or fibers (called whiskers), but SiC toxicity has been poorly

investigated and the few reported data are controversial, making it difficult to draw clear conclusions. For instance, it has been shown that SiC particles do not induce harmful effects on tissues, suggesting SiC material is quite biologically inert[10, 11]. Conversely, other studies have suggested potential adverse effects induced by SiC. *In vivo* exposure to whiskers triggers lung inflammation[13], granulomas[14], bronchoalveolar hyperplasia and severe lung fibrotic changes[15], while micro-scaled SiC particles were associated to oxidant stress, genotoxic and carcinogenic effects[16-18]. Moreover, *in vitro* responses are characterized by significant reactive oxygen species (ROS) generation[17], cytotoxic and genotoxic effects[17,18], and stimulation of pro-inflammatory cytokines production like TNF-α[13, 18]. These results mainly underline a potential impact of SiC particle shape on the biological activity, because SiC whiskers (*i.e.* long and rigid rods of SiC having aspect ratios that exceed 10:1) induce more important biological activity compared to isotropic SiC particles[17].

Recent *in vitro* studies, specifically devoted to SiC nanoparticles, highlighted an accumulation of particles in A549 lung epithelial cells, a major cell oxydo-reduction status disturbance and DNA damage[19, 20]. Another study clearly showed the impact of the nanoparticle surface area and the nature of crystalline phases (α-SiC *vs.* β-SiC) on the TNF-α production, the influence of the iron content at the nanoparticle surface on the free radical release, and the surface oxidation on the cellular H_2O_2 production[21]. Thus, these authors proposed to reconsider SiC nanoparticles biocompatibility and to handle SiC nanoparticles with caution until more toxicological information is available.

In this context, it seems crucial to investigate more deeply the interactions between SiC industrial powders and living cells. Consequently, we conducted a multidisciplinary study in order to examine the impact of the physico-chemical parameters of SiC industrial particles on the *in vitro* cellular responses. As a matter of fact, a main challenge is the lack of comprehensive data to assess the potential toxicity of a SiC industrial particle collection

regarding different sizes, oxidation state of surfaces, crystallographic structure or chemical composition. These complementary physico-chemical and biological approaches may be particularly suitable for safer by design development.

To reach this objective we studied 5 polydisperse industrial SiC powders produced by the Acheson process in industrial plants (coarse powders SiC A1/A3/A4; fine SiC A2/A5 powders). Their physico-chemical features were first characterized: crystallographic structure, C/Si atomic ratio (*i.e.* silicon or carbon excess), surface oxidation state (*i.e.* the formation of a silica or oxycarbide layer around the SiC grain) and the presence of iron impurities on particle surface. *In vitro* toxicological assessment was then carried out using macrophages (RAW 264.7, a cultured murine macrophage cell line widely used in toxicological studies due to its phagocytic capacity). The cytotoxicity (LDH release), the pro-inflammatory response (TNF-α production), and the oxidative stress (H_2O_2 specific production) were assessed. Finally, the particle potential to release free radical species was evaluated in cell free condition. Two radical-generating mechanisms have been examined: i) $HO^{\bullet}$ radical generation in the presence of hydrogen peroxide. This test mimics the contact with lysosomal fluids where H_2O_2 is released following particle phagocytosis by alveolar macrophages; and ii) $COO^{\bullet-}$ from the formate ion, used as a model target molecule for homolytic cleavage of a carbon-hydrogen bond in several organic molecules and biomolecules.

Finally, to better investigate the impact of the surface nature of the particles on cellular responses, coarse SiC A1 and fine SiC A2 powders underwent an oxidative thermal post-treatment at different temperatures (650°C, 750°C, 850°C, 1400°C). Biological activity was then assessed.

Experimental procedures

Collection of manufactured SiC powders

In this study, the 5 SiC industrial powders used (SiC A1 to SiC A5) were produced by the Acheson process, and collected in industrial plants. SiC A1 and A4 particles correspond to coarse α-SiC microparticles and are mainly used for abrasives and refractory applications. SiC A2 and A5 particles correspond to fine α-SiC microparticles and are mainly used for particulate filters and technical ceramics. SiC A3 powder can be considered as a metallurgical impure powder with no particular industrial application, but this sample is representative of airborne dusts that can be disseminated and inhaled in the work environment. Only alveolar fractions of SiC powders have been studied using a vertical elutriator[22]. This fraction corresponds to particles lower than 10 μm contained in raw powders.

Oxidative thermal post-treatment

SiC A1 and A2 powders were submitted to increasing thermal treatments under air atmosphere. SiC grains monolayer bed was laid out in a crucible (AluSIK-99 ZA) in the middle of a tubular furnace (Nabertherm RHT08/17), and exposed for 2 hours at the following temperatures: 650°C, 750°C, 850°C and 1400°C.

SiC powders characterization

SiC physico-chemical characteristics were investigated using the following methods. Particles morphology was observed using a field-emission scanning electron microscope (JEOL JSM-6500F). The specific surface area (SSA, m^2/g) was determined by N_2 adsorption at 77K after out-gassing 2h at 200°C (Micromeritics ASAP 2000), using the Brunauer-Emmet-Teller (BET) method. Particle size distribution and crystalline structure were determined respectively by laser granulometry (Malvern, Mastersizer 2000) and X-ray diffractometry with a semi-quantitative analysis by Rietveld Method (Siemens Kristalloflex diffractometer D8). Particle diameter (BET size, μm) was calculated as BET size = 6000 / (ρ × SSA). Concentration of constitutive elements (Si, C) and impurities (especially iron and aluminum)

was deduced from elemental chemical analysis by inductively coupled plasma spectrometry (ICP, Jobin-Yvon Activa). The oxidation state of SiC powders (silica, quartz, cristobalite, oxycarbide layer around SiC grains) was characterized by surface analysis using X-ray photoelectron spectroscopy (XPS, Thermo VG Thetaprobe) and X-ray diffractometry.

Free radical release

Electron paramagnetic resonance (EPR) using the spin-trapping technique[23] was used to evaluate SiC particle potential to generate free radicals (radical hydroxyl $HO^{\bullet}$, radical carboxyl $COO^{\bullet -}$) in aqueous suspensions. Each powder (45 mg) was suspended in a buffered solution (0.5M potassium phosphate buffer, pH 7.4) 0.15M of DMPO (5-5'-dimethyl-1-pirroline-Noxide). The reaction was started by adding the substrate molecule respectively hydrogen peroxide (1.0M) or sodium formate (1.0M) to the SiC particle suspension. The radical yield was progressively measured in a 50 µl aliquot of the suspension up to one hour. Radical species release was monitored in the suspension by EPR spectroscopy (Miniscope 100 EPR spectrometer, Magnettech). The instrument settings were as follows: microwave power 10 mW; modulation 1000 mG; scan range 120 G; centre of field approximately 3345 G. The use of internal standard (Mn) enabled radical activity quantification. Each experiment was repeated three times.

Cell line and culture conditions

The RAW 264.7 cell line derived from murine peritoneal macrophages transformed by the AML Virus (Abelson Murine Leukemia Virus) was provided by the ATCC Cell Biology Collection (Promochem LGC, Molsheim). Cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM, Invitrogen), complemented with 10% fetal calf serum (FCS, Invitrogen), 1% penicillin-streptomycin (penicillin 10 000 units/mL, streptomycin 10mg/mL; Sigma-

Aldrich, Saint-Quentin Fallavier, France) and incubated at 37°C under a 5% carbon dioxide humidified atmosphere.

Cell viability was determined by trypan blue dye exclusion (FDA, Sigma). For each experiment, as described by Leclerc et al.[24], cells were prepared in 96-well plates (100 000 cells/well for TNF-α and LDH assays, and 300 000 cells/well for H_2O_2 parameter) in 25 µL of complete DMEM (DMEMc). Suspensions of SiC powders were prepared in 75 µL of DMEMc which were added to the culture and incubated for 90 min at 37°C in a 5% CO_2 atmosphere. Different doses were tested: 15, 30, 60 and 120 µg of particles per mL of culture medium. Negative and positive controls of toxicity, corresponding to the cells incubated alone or incubated with DQ12 quartz respectively[25] were included. Three independent experiments were performed for each condition.

Cytotoxicity assays

The activity of the lactate dehydrogenase (LDH) released from cells with damaged membranes in the culture supernatant was assessed after a 24h incubation of cells with SiC particles. The CytoTox-ONE™ Homogeneous Membrane Integrity Assay (Promega, Charbonnières les bains, France) was used according to the manufacturer's instructions. Detection was performed using a fluorometer (Fluoroskan Ascent, Thermolabsystems), with excitation/emission wavelengths set at 530/590 nm. The activity of the released LDH was reported to that of total cellular LDH (measured after the lysis of control cells) and was expressed as a percentage of the control.

Pro-inflammatory response

Tumor Necrosis Factor alpha (TNF-α) production was assessed in the culture supernatant after a 24h incubation of cells with SiC particles. A commercial ELISA Kit (Quantikine® Mouse TNF-α Immunoassay, R&D Systems, Lille, France) was used according to the manufacturer's instructions. The optical density of each well was determined using a microplate reader (Multiskan RC, Thermolabsystems, Helsinki, Finland) set at 450 nm. A standard curve was established and results were expressed in pg/mL of TNF-α.

Oxidative stress with H_2O_2 production

Acute oxidative stress was assessed by the production of H_2O_2 after 90 min interaction between the cells and the different types of SiC powders. Hydrogen peroxide H_2O_2 production was measured according to the protocol of De la Harpe and Nathan[26]. Briefly, KRPG (Krebs-Ringer phosphate glucose) buffer, containing a mixture of scopoletin (30 µM), NaN_3 (1 mM) and horseradish peroxidase (1 unit pupurogallin/ml HPO) was added to cells. Over a 90 min period, fluorimetric determination (355/460 nm excitation/emission wavelengths) of scopoletin oxidation, catalyzed by horseradish peroxidase was measured (Fluoroskan Multiskan). Results were expressed as the amount of H_2O_2 released by cells (nmol $H_2O_2/10^6$ MA).

Statistical analysis

Results are expressed as means of three independent experiments. Statistical significance was declared when $p<0.05$ as determined using a Student test.

Results and discussion

A comprehensive physico-chemical characterization of 5 industrial SiC powders was carried out, followed by the *in vitro* evaluation of their biological toxicity.

Physico-chemical data are presented in Table 1 with an illustration of the morphological aspect of SiC particles given by Figure 1. SiC powders were polydispersed and exhibited an irregular-shaped morphology characterized by a smooth surface with sharp ridges and the presence of submicronic particles fixed on larger particles which is a common feature of powders synthesized by the Acheson process (Figure 1).

They could be classified either as 2.5 μm coarse particles (SiC A1 and A4) or 0.5 μm fine particles (SiC A2 and A5). SiC A3 was kept apart because of a higher size (6 μm), the presence of crystallized silica and its very high metallic impurities content (iron about 20 000 ppm compared to 2500 ppm for other powders). Except SiC A3 showing a very high crystallized silica content and only 65% of α-SiC phases, the other powders (A1, A2, A4 and A5) correspond to pure α-SiC phases (> 99%), with very similar features in terms of C/Si ratio around 1 and crystallographic structure (principally with SiC-6H polytype and SiC-4H and SiC-15R <10%). By contrast SiC A3 powder has a 1.66 C/Si ratio associated with high oxygen and free carbon levels (%O ≈ 6 %, $\%C_{Free}$ ≈ 17.5 %) and the presence of crystallized silica (9% of quartz and 5% of cristobalite).

Biological effects of SiC powders concerning cytotoxicity, pro-inflammatory effect and oxidative stress are reported in Figures 2, 3 and 4 respectively.

Surprisingly all the 5 SiC powders were found to be globally not cytotoxic (Figure 2). However, even if no dose-dependent response was observed in the LDH release, some LDH values obtained for the SiC A4 and A5 powders were significantly higher than that of control cells. SiC A4 and A5 powders could therefore have a cytotoxic potential compared to powders SiC A1, A2 and A3, without a specific relationship to a physico-chemical parameter. The positive control (DQ12 quartz) clearly showed a significantly enhanced and dose-dependent LDH release.

All particles induced a moderate inflammation (Figure 3), except SiC A3 which generated a strong TNF-α production (around 3000 pg/ml at 120 µg dose for example), even higher than that induced by the DQ12 quartz (around 2400 pg/ml at the same dose). This SiC A3 atypical profile could be explained on the one hand by the presence of crystallized silica (quartz and cristobalite) with high free carbon levels, and on the other hand by its high content of iron impurities (Table 3)[27–29].

It has been well described in the literature that there is a relationship between the presence of surface transition metal ions (especially iron) and cellular responses[23,30,31], especially for free radical generation[32–35]. Indeed iron is known to be a catalyst in the formation of several reactive oxygen species[36], particularly the hydroxyl radical can be generated through the Fenton reaction ($Fe^{2+}+H_2O_2 \rightarrow Fe^{3+}+OH^-+HO^\bullet$) when ferrous iron (Fe[II]) are converted into ferric iron (Fe[III])[37].

Free radical generation and H_2O_2 cellular production have been employed to assess the ability of SiC particles to induce oxidative stress. Free radical generation in cell free medium only depends on the presence of reactive sites (eg redox active metal ions) at the particle surface. Conversely, H_2O_2 production in macrophage cells following the phagocytic activity is the result of a complex process. So, the H_2O_2 production induced by fine SiC A2 and A5 particles, regardless the dose was much higher than that triggered by the coarse SiC A1 / A4 or A3 powders and even more elevated than that caused by the DQ12 quartz (Figure 4). These results clearly demonstrate the impact of particle size on some cellular responses, especially on oxidative stress. More specifically, this impact of particle size can be explained by a linear relationship between the H_2O_2 production and the particle surface ($y = 15.9x + 21.5$, where y = H_2O_2 production in nmol/10^6 cells and x = particle surface in cm²; $R^2 = 0.9$). This observation is in agreement with results previously published in the literature for SiC or other particles like TiO_2 and carbon black. Several studies reported that small particles induced a

higher reactive oxygen species production than the bigger ones due to biological activity piloted by the available particle surface area[38–44]. The present results also corroborate a previous study performed with nanosized SiC particles[21].

To investigate the ability of SiC to generate particle derived free radical species (not at the cellular level), $HO^{\bullet}$ and $COO^{\bullet-}$ production was assessed in cell free condition by EPR spectroscopy (Figure 5). All particles were able to release free radicals and were more effective in $COO^{\bullet-}$ than in $HO^{\bullet}$ radical generation. Free radical production increased with particle size, it was 3-4 fold higher for the coarse SiC A1 / A4 particles or SiC A3 than for fine SiC A2 /A5 particles. $HO^{\bullet}$ and $COO^{\bullet-}$ release did not parallel the H_2O_2 production observed in macrophage cells. This is not surprising given the different test conditions. Free radical generation in cell free environment, as opposed to ROS generation in a cellular test, is determined only by the intrinsic characteristics of the particle surface, e.g. surface composition or crystalline state. Note that the iron content (Table 3) is higher in the coarse particles than in the fine ones and this may account for the high surface reactivity of SiC A1/A3/A4. Note also that the SiC A3, which released the largest amount of radical species, exhibited a partially crystallized surface silica layer.

To further investigate the role of surface oxidation state, two SiC powders (A1 and A2) were submitted to increasing thermal treatments (from 600 to 1400°C). Oxidative thermal treatments lead to surface modifications characterized by an increase of the silica layer thickness at 1400°C, as illustrated in Figure 6[45–48]. At this temperature Fe^{2+} is also converted into Fe^{3+}.

The physico-chemical characterization of the oxidized SiC A1 and SiC A2 powders revealed no significant changes in the morphology or specific surface area of the particles, except at 1400°C where the morphology was strongly modified presenting a grain aspect mainly characterized by less sharp ridges and more rounded edges. SiC particles were progressively

covered with a surface amorphous SiO_2 layer which was transformed into cristobalite silica at 1400°C (Table 2 and Figure 6). This observation was correlated with an increased oxide layer, judging by the silica evolution and a silicon carbide peak decrement (data not shown). At 1400°C, oxidized SiC A1 and SiC A2 showed both a SSA and a C/Si ratio decrement (Table 2).

The biological activity assessment after thermal treatments is presented in Figures 7 to 10. A significant development of cytotoxicity was observed for oxidized SiC A2 compared to that of the original SiC A2 powder, whatever the temperature (Figure 7). On the contrary, oxidative treatments had no impact on the cytotoxicity of SiC A1, irrespective of the temperature. At an equivalent dose, the LDH release for the fine oxidized SiC A2 powder was higher than for the coarse oxidized SiC A1 powder, suggesting that fine particles exhibiting a higher surface could be more easily oxidized, which resulted in a higher cytotoxicity.

Similarly, oxidized SiC A1 showed no significant induction of inflammation compared to the negative control and unoxidized SiC A1 (Figure 8), whereas oxidized SiC A2 (from 650-850°C) exhibited a TNF-α production drastically higher than that induced by SiC A2 particles. However, it is interesting to note that both oxidized SiC A1 and SiC A2 particles treated at 1400°C, despite the presence of a silica cristobalite layer around SiC grains, presented a TNF-α production equivalent to that of the negative control and slightly lower than their original counterparts. Some studies indicate that cristobalite originated by heating quartz at high temperature (>1300°C), as well heated cristobalite, exhibits higher hydrophobicity and lower amount of surface radicals[23, 49] if compared to other cristobalite samples, such as those originated from diatomaceous earth heated at 1000°C. Heated cristobalite also exhibits a lower toxicity in cell free tests[28]. This may partially explain the limited pro-inflammatory effect of industrial SiC powders treated at 1400°C.

Oxidation of SiC A1 did not influence the H_2O_2 production (Figure 9). Although oxidized SiC A2 exhibited a H_2O_2 generation higher than that of the positive control (irrespective of the temperature), it remained in the same range as the original SiC A2 powder, suggesting that the production of H_2O_2 was not correlated with oxidation and iron content (Table 3). The different behavior between oxidized SiC A1 and A2 could be once again explained by a size effect.

The increasing amorphous surface layer and progressive conversion of Fe^{2+} to Fe^{3+} following oxidation at low temperatures (<850°C) are likely responsible for the decrease in free radical generation (Figure 10). The decrease was more noticeable for carboxyl than for hydroxyl radicals. Although the mechanism of $COO^{\bullet-}$ generation is not still fully clarified, the reduction of reactivity subsequent to oxidation of active surface sites has been observed also in other particulates

However, SiC A1 and A2 oxidized at 1400°C showed an increased capacity to generate an EPR signal, mainly for $HO^{\bullet}$ but also for $COO^{\bullet-}$ radicals even if for this latter, the effect was much less pronounced (Figure 10). At this treatment temperature, two correlations can be established: between $HO^{\bullet}$, $COO^{\bullet-}$ radicals and crystallization on the one hand (cristobalite > amorphous SiO_2) and between $HO^{\bullet}$, $COO^{\bullet-}$ radicals and size on the other hand (coarse oxidized SiC A1 > fine oxidized SiC A2, Table 3). These observations were in agreement with previous literature studies describing specifically a more important toxicity associated with a higher radical potential for crystallized silica compared to amorphous SiO_2[28, 32]. Finally, we have observed as for original SiC A1 and A2 powders an opposite trend between cellular oxidative stress (H_2O_2 production) and free radical generation in cell free conditions. The EPR radical potential was temperature dependent and directly related to the silica phase nature: amorphous SiO_2 at 650,750 and 850°C compared to cristobalite SiO_2 at 1400°C (Table 2).

Conclusion

The toxicity of particles is known to be closely related to their physico-chemical properties (size, shape, surface area, chemical composition, etc.). In order to deepen such a relationship, a thorough physico-chemical characterization of different SiC powders produced by the Acheson process and collected at workplace was carried out, in association with an *in vitro* evaluation of their biological activity. Table 3 summarizes the relationships put in evidence in the present study.

We have thereby demonstrated that all types of SiC particles tested here did not induce any significant cytotoxic effect. Three parameters that had an impact on the *in vitro* biological response were identified: the particle size, the presence of iron impurities at the particle surface and the oxidation state of the particles.

The particle size was found to have an impact on SiC powders related to oxidative stress. This parameter seems to have also an influence on cytotoxicity and oxidative stress for oxidized SiC powders due to thermal treatment. The presence of iron metallic impurities at the surface of the particles mainly stimulated a pro-inflammatory response, which was abolished when amorphous SiO_2 was transformed into cristobalite at 1400°C. The oxidation surface modifications by this 1400°C thermal treatment seemed to also have a deep impact on $HO^{\bullet}$ and $COO^{\bullet-}$ radical generation.

Taken together these results support the conclusion that manufactured SiC powders do not induce similar levels of toxicity, underlining the need to systematically evaluate the biological activity in relation to the particles physico-chemical features through case by case studies. Furthermore, the modification of surface powders has clearly emphasized the major importance of surface oxidation state, in addition to more usual physico-chemical features such as size, SSA, shape or chemical composition.

This approach combining systematically physico-chemical characterization and biological assessment is particularly recommended in the evaluation of occupational risks in industrial environments or in the context of "safer by design" principles[50]. By tightly controlling the physico-chemical features of the industrial SiC particles pro-inflammatory effect and oxidative stress could be limited. A particularly interesting observation is that SiC toxicity seems to be modulated by the oxidized external layer around SiC grains. It opens new perspectives in the preparation of industrial powders as the prevention of potential hazard seems easier at the design stage than during the manufacturing processes or at the customer use level.

Funding information

This work was supported by the French Ministry of Industry and an industrial partner.

Abbreviations

BET method : Brunauer-Emmet-Teller method

$COO^{\bullet -}$: carboxyl radical

DMEM : Dulbecco's Modified Eagle's Medium

EPR : Electron paramagnetic resonance

FCS : fetal calf serum

$HO^{\bullet}$: hydroxyl radical

H_2O_2 : hydrogen peroxide

ICP : inductively coupled plasma spectrometry

ROS : Reactive Oxygen Species

SiC : Silicon carbide

SSA : specific surface area

TNF-α : Tumor Necrosis Factor

XPS : X-ray photoelectron spectroscopy

References

(1) Acheson, E. G. (1893, February 28) Production of artificial crystalline carbonaceous materials.

(2) Pham-Huu, C., Bouchy, C., Dintzer, T., Ehret, G., Estournes, C., and Ledoux, M. J. (1999) High surface area silicon carbide doped with zirconium for use as catalyst support. Preparation, characterization and catalytic application, *Appl. Catal. A-Gen. 180*, 385–397.

(3) Winslow, C.-E. A., Greenburg, L., and Greenberg, D. (1919) The Dust Hazard in the Abrasive Industry, *Public Health Reports (1896-1970) 34*, 1171–1187.

(4) Bruusgaard A. (1948) Pneumoconiosis in silicon carbide workers., *Proceedings of the 9th International Congress on Industrial Medicine. London, United Kingdom: Wright Briston.* 676–81.

(5) Hayashi, H., and Kajita, A. (1988) Silicon carbide in lung tissue of a worker in the abrasive industry, *Am. J. Ind. Med. 14*, 145–155.

(6) Osterman, J. W., Greaves, I. A., Smith, T. J., Hammond, S. K., Robins, J. M., and Thériault, G. (1989) Work related decrement in pulmonary function in silicon carbide production workers, *Br J Ind Med 46*, 708–716.

(7) Funahashi, A., Schlueter, D. P., Pintar, K., Siegesmund, K. A., Mandel, G. S., and Mandel, N. S. (1984) Pneumoconiosis in workers exposed to silicon carbide, *Am. Rev. Respir. Dis. 129*, 635–640.

(8) Massé, S., Bégin, R., and Cantin, A. (1988) Pathology of silicon carbide pneumoconiosis, *Mod. Pathol. 1*, 104–108.

(9) Dufresne, A., Sébastien, P., Perrault, G., Massé, S., and Bégin, R. (1992) Pulmonary clearance of fibrous and angular SiC particulates in the sheep model of pneumoconiosis, *Ann Occup Hyg 36*, 519–530.

(10) Bruch, J., Rehn, B., Song, H., Gono, E., and Malkusch, W. (1993) Toxicological investigations on silicon carbide. 1. Inhalation studies, *Br J Ind Med 50*, 797–806.

(11) Bruch, J., Rehn, B., Song, W., Gono, E., and Malkusch, W. (1993) Toxicological investigations on silicon carbide. 2. In vitro cell tests and long term injection tests, *Br J Ind Med 50*, 807–813.

(12) Bruch, J., and Rehn, B. (1996) Relevant differences in pathogenicity of nuisance dusts; model investigations on samples of silicon carbide dusts, *Exp. Toxicol. Pathol. 48*, 477–480.

(13) Cullen, R. T., Miller, B. G., Davis, J. M., Brown, D. M., and Donaldson, K. (1997) Short-term inhalation and in vitro tests as predictors of fiber pathogenicity, *Environ. Health Perspect. 105 Suppl 5*, 1235–1240.

(14) Vaughan, G. L., Trently, S. A., and Wilson, R. B. (1993) Pulmonary response, in vivo, to silicon carbide whiskers, *Environ. Res. 63*, 191–201.

(15) Akiyama, I., Ogami, A., Oyabu, T., Yamato, H., Morimoto, Y., and Tanaka, I. (2007) Pulmonary effects and biopersistence of deposited silicon carbide whisker after 1-year inhalation in rats, *Inhal Toxicol 19*, 141–147.

(16) Cichy, B. (1989) Pathogenic and carcinogenic properties of silicon carbide, *Pol J Occup Med 2*, 87–96.

(17) Svensson, I., Artursson, E., Leanderson, P., Berglind, R., and Lindgren, F. (1997) Toxicity in vitro of some silicon carbides and silicon nitrides: whiskers and powders, *Am. J. Ind. Med. 31*, 335–343.

(18) Allen, M., Butter, R., Chandra, L., Lettington, A., and Rushton, N. (1995) Toxicity of particulate silicon carbide for macrophages, fibroblasts and osteoblast-like cells in vitro, *Biomed Mater Eng 5*, 151–159.

(19) Barillet, S., Jugan, M.-L., Laye, M., Leconte, Y., Herlin-Boime, N., Reynaud, C., and Carrière, M. (2010) In vitro evaluation of SiC nanoparticles impact on A549 pulmonary cells: cyto-, genotoxicity and oxidative stress, *Toxicol. Lett. 198*, 324–330.

(20) Barillet, S., Simon-Deckers, A., Herlin-Boime, N., Mayne-L'Hermite, M., Reynaud, C., Cassio, D., Gouget, B., and Carriere, M. (2010) Toxicological consequences of TiO2, SiC nanoparticles and multi-walled carbon nanotubes exposure in several mammalian cell types: an in vitro study, *J. Nanopart. Res. 12*, 61–73.

(21) Pourchez, J., Forest, V., Boumahdi, N., Boudard, D., Tomatis, M., Fubini, B., Herlin-Boime, N., Leconte, Y., Guilhot, B., Cottier, M., and Grosseau, P. (2012) In vitro cellular responses to silicon carbide nanoparticles: impact of physico-chemical features on pro-inflammatory and pro-oxidative effects, *J. Nanopart. Res. 14*.

(22) Boumahdi, N. (2009) Approche pluridisciplinaire de l'étude de l'activité biologique de particules fines, Thèse de doctorat, École nationale supérieure des mines, Saint-Etienne, France.

(23) Fubini, B., Mollo, L., and Giamello, E. (1995) Free radical generation at the solid/liquid interface in iron containing minerals, *Free Radic. Res. 23*, 593–614.

(24) Leclerc, L., Boudard, D., Pourchez, J., Forest, V., Sabido, O., Bin, V., Palle, S., Grosseau, P., Bernache, D., and Cottier, M. (2010) Quantification of microsized fluorescent particles phagocytosis to a better knowledge of toxicity mechanisms, *Inhal Toxicol 22*, 1091–1100.

(25) Bruch, J., Rehn, S., Rehn, B., Borm, P. J. A., and Fubini, B. (2004) Variation of biological responses to different respirable quartz flours determined by a vector model, *Int J Hyg Environ Health 207*, 203–216.

(26) De la Harpe, J., and Nathan, C. F. (1985) A semi-automated micro-assay for H2O2 release by human blood monocytes and mouse peritoneal macrophages, *J. Immunol. Methods 78*, 323–336.

(27) IARC. (1996) IARC Monograph on the Evaluation of Carcinogenic Risks to Humans., p 578, Printing Processes and Printing Inks, Carbon Black and Some Nitro Compounds, IARC, Lyon, France.

(28) Fubini, B., Zanetti, G., Altilia, S., Tiozzo, R., Lison, D., and Saffiotti, U. (1999) Relationship between surface properties and cellular responses to crystalline silica: studies with heat-treated cristobalite, *Chem. Res. Toxicol. 12*, 737–745.

(29) Governa, M., Amati, M., Fenoglio, I., Valentino, M., Coloccini, S., Bolognini, L., Carlo Botta, G., Emanuelli, M., Pierella, F., Volpe, A. R., Astolfi, P., Carmignani, M., and Fubini, B. (2005) Variability of biological effects of silicas: different degrees of activation of the fifth component of complement by amorphous silicas, *Toxicol. Appl. Pharmacol. 208*, 68–77.

(30) Pritchard, R. J., Ghio, A. J., Lehmann, J. R., Winsett, D. W., Tepper, J. S., Park, P., Gilmour, M. I., Dreher, K. L., and Costa, D. L. (1996) Oxidant generation and lung injury

after particulate air pollutant exposure increase with the concentrations of associated metals, *Inhal. Toxicol. 8*, 457–477.

(31) Aust, A. E., Lund, L. G., Chao, C. C., Park, S. H., and Fang, R. H. (2000) Role of iron in the cellular effects of asbestos, *Inhal. Toxicol. 12*, 75–80.

(32) Fubini, B., and Hubbard, A. (2003) Reactive oxygen species (ROS) and reactive nitrogen species (RNS) generation by silica in inflammation and fibrosis, *Free Radic. Biol. Med. 34*, 1507–1516.

(33) Fenoglio, I., Prandi, L., Tomatis, M., and 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.

(34) Van Maanen, J. M., Borm, P. J., Knaapen, A., Van Herwijnen, M., Schilderman, P. A., Smith, K. R., Aust, A. E., Tomatis, M., and Fubini, B. (1999) In vitro effects of coal fly ashes: hydroxyl radical generation, iron release, and DNA damage and toxicity in rat lung epithelial cells, *Inhal Toxicol 11*, 1123–1141.

(35) Turci, F., Tomatis, M., Lesci, I. G., Roveri, N., and Fubini, B. (2011) The iron-related molecular toxicity mechanism of synthetic asbestos nanofibres: a model study for high-aspect-ratio nanoparticles, *Chemistry 17*, 350–358.

(36) Pierre, J. L., and Fontecave, M. (1999) Iron and activated oxygen species in biology: the basic chemistry, *Biometals 12*, 195–199.

(37) Halliwell, B., and Gutteridge, J. M. (1992) Biologically relevant metal ion-dependent hydroxyl radical generation. An update, *FEBS Lett. 307*, 108–112.

(38) Oberdörster, G., Ferin, J., and Lehnert, B. E. (1994) Correlation between particle size, in vivo particle persistence, and lung injury, *Environ. Health Perspect. 102 Suppl 5*, 173–179.

(39) Oberdorster, G. (2000) Toxicology of ultrafine particles: in vivo studies, *Philos. Trans. R. Soc. Lond. Ser. A-Math. Phys. Eng. Sci. 358*, 2719–2739.

(40) Oberdörster, G., Oberdörster, E., and Oberdörster, J. (2005) Nanotoxicology: an emerging discipline evolving from studies of ultrafine particles, *Environ. Health Perspect. 113*, 823–839.

(41) Brown, D. M., Wilson, M. R., MacNee, W., Stone, V., and Donaldson, K. (2001) Size-dependent proinflammatory effects of ultrafine polystyrene particles: a role for surface area and oxidative stress in the enhanced activity of ultrafines, *Toxicol. Appl. Pharmacol. 175*, 191–199.

(42) Höhr, D., Steinfartz, Y., Schins, R. P. F., Knaapen, A. M., Martra, G., Fubini, B., and Borm, P. J. A. (2002) The surface area rather than the surface coating determines the acute inflammatory response after instillation of fine and ultrafine TiO2 in the rat, *Int J Hyg Environ Health 205*, 239–244.

(43) Donaldson, K., Brown, D., Clouter, A., Duffin, R., MacNee, W., Renwick, L., Tran, L., and Stone, V. (2002) The pulmonary toxicology of ultrafine particles, *J. Aerosol Med.-Depos. Clear. Eff. Lung 15*, 213–220.

(44) Fubini, B. (2007) Particules ultra-fines□: propriétés physicochimiques et activité biologique. In *Les nanoparticules: Un enjeu majeur pour la santé au travail ?*, pp 499–529, EDP Sciences, France.

(45) Deal, B. E., and Grove, A. S. (1965) General Relationship for the Thermal Oxidation of Silicon, *Journal of Applied Physics 36*, 3770–3778.

(46) Filipuzzi, L. (1991) Oxydation des composites SIC/SIC et de leurs constituants : approche expérimentale, modélisation et influence sur le comportement mécanique, Thèse de doctorat, France.

(47) Shi, Z. L., Lee, J., Zhang, D., Lee, H., Gu, M. Y., and Wu, R. J. (2001) The passive-oxidized behavior of SiC particles and their jointing characteristics, *J. Mater. Process. Technol. 110*, 127–131.

(48) Das, D., Farjas, J., and Roura, P. (2004) Passive-oxidation kinetics of SiC microparticles, *J. Am. Ceram. Soc. 87*, 1301–1305.

(49) Fubini, B., Bolis, V., Cavenago, A., and Volante, M. (1995) Physicochemical properties of crystalline silica dusts and their possible implication in various biological responses, *Scand J Work Environ Health 21 Suppl 2*, 9–14.

(50) Morose, G. (2010) The 5 principles of "Design for Safer Nanotechnology", *J. Clean Prod. 18*, 285–289.