# The Pathogenicity of Crystalline Silica
## *crystallinity or surface disorder?*


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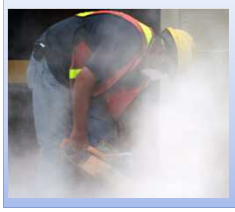
## Background and objectives



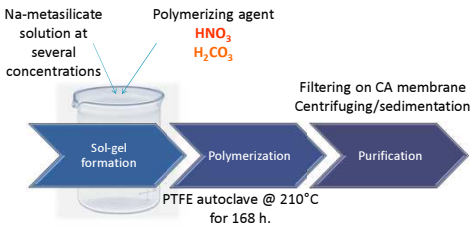
Occupational exposure to some quartz dusts is associated to severe lung diseases (*i.e.* silicosis, lung cancer and autoimmune pathologies) *(IARC, 2012)*, but the hazard posed by quartz particles varies from one to the other source *(Donaldson&Borm, 1998)*. What imparts pathogenicity to any single quartz source is in part still unclear. Crystallinity and various surface features are implied in toxicity *(Fubini, 1998)*.

Quartz dusts used so far for toxicological studies have been obtained from fragmentation, a process which affects crystallinity and yields dusts with unpredictable and variable surface states, thus hindering a reliable correlation between surface features and toxic effects. To clarify the role of crystallinity and surface state in silica pathogenicity we have:

1. synthesized quartz crystals in respirable size (aerodynamic Ø < 5 µm) *(Pastero et al., 2016)* and induced fractures via controlled ball-milling
2. investigated particle toxicity in *in vitro* tests *(Turci et al., 2016)*
3. Assessed the physico-chemical features of the particles to understand the molecular determinants governing cell toxicity *(Turci et al., 2016)*

## 1. Synthesis of submicron and micron quartz crystals with intact surfaces

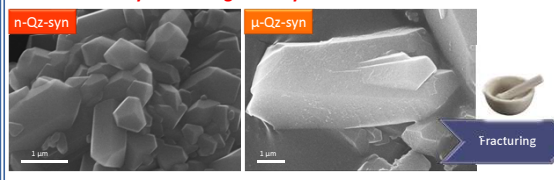
### Synthetic routes



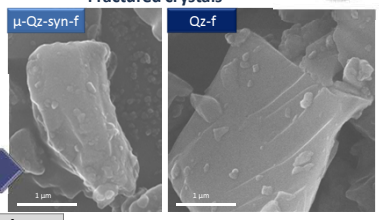

We obtained two different sample of quartz through an innovative and easy to perform sol-gel hydrothermal method which employs Na-metasilicate solution polymerized using simple mineral acids ($HNO_3$, $H_2CO_3$), in order to keep the chemical complexity of the gel as low as possible. Quartz can grow in short runs and in mild (T, p) conditions.

### Quartz morphology and size

**Synthetic as-grown crystals**




**Fractured crystals**




| Acronym | Crystal type | Particle diameter 10th %ile (nm)§ | Particle diameter 50th %ile (nm)§ | Particle diameter 90th %ile (nm)§ | Surface area (m²/g)† |
|---|---|---|---|---|---|
| n-Qz-syn | Synthetic as-grown crystals in submicron size | 174 | 276 | 408 | 6.2 |
| µ-Qz-syn | Synthetic as-grown crystals in micron size | 405 | 904 | 2520 | 0.3 |
| µ-Qz-syn-f | Fractured micron size synthetic crystals | 409 | 914 | 1960 | 9.5 |
| Qz-f | Mineral quartz dust (positive control) | 285 | 704 | 1290 | 6.1 |

§Differential Centrifugal Sedimentation (DCS) †Kr-BET method

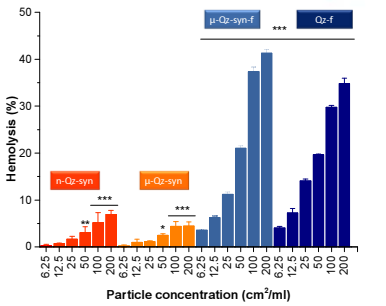
**FE-SEM images** show the morphological differences among the two preparations of synthetic as-grown quartz crystals (n-Qz-syn and µ-Qz-syn) and the fractured ones (µ-Qz-syn-f, Qz-f) obtained by ball-milling. As-grown quartz are characterized by flat, regular, and smooth surfaces. Mechanical milling induces the formation of conchoidal fractures commonly observed on industrial quartz dusts.

**Table**: Size and specific surface area of the quartz studied. The synthetic procedure allowed to obtain two quartz samples different for size and SSA, one in submicron size (100-400 nm) (n-Qz-syn), the other in micron size (up to 2500 nm) (µ-Qz-syn).

## 2. In vitro studies

### Hemolytic activity

(Red blood cells as model of cell membranes)

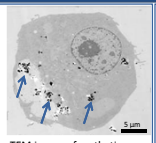



Increasing concentrations of quartz crystals were incubated with purified human red blood cells. A modest lytic effect towards RBCs was observed upon incubation with as-grown quartz crystals, while a remarkable dose-dependent lysis was induced by fractured quartz.
*(ANOVA +Tukey's) *p< 0.05, **p< 0.01 and ***p< 0.001 vs control*

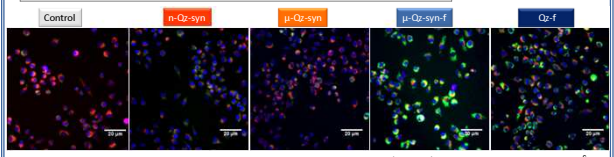
### Cell toxicity in macrophages

(Raw 264.7, 24h exposure, 100 µg/ml, *vs* control not exposed)

| Sample | Cell death§ *(cell count)* | Necrosis† *(nuclear size/intensity)* | Lysosomal stress§ *(acidification)* |
|---|---|---|---|
| n-Qz-syn | - | - | - |
| µ-Qz-syn | - | - | - |
| µ-Qz-syn-f | - | ++ | +++ |
| Qz-f | ++ | ++ | ++ |



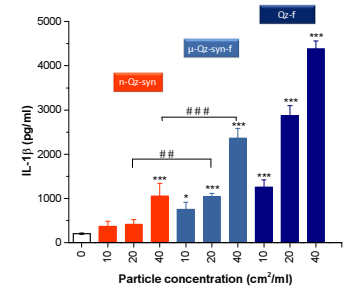
TEM image of synthetic quartz uptaken by macrophages. Both as-grown and fractured quartz were efficiently internalized into phagolysosomes.




High Content Analysis. Murine macrophages were exposed for 24 h to medium (Control) or increasing doses of quartz. §Cell count (number of **Hoechst** stained nuclei → blue fluo), †nuclear size and intensity (average object area of Hoechst and intensity), ‡ lysosomal acidification (**Lysotracker Green** intensity → green fluo) were measured. As-grown quartz crystals were internalized but resulted inactive at the highest dose (100 µg/ml) for all the cytotoxicity parameters investigated, while fractured quartz induced significant cell stress. Mitochondrial membrane potential (**TMRM** intensity → red fluo).

### Inflammatory activity

(IL-1β release from THP-1macrophages, 6h exposure)




THP-1 macrophages were exposed for 6 h to increasing concentrations of quartz. Secretion of IL-1β was measured in cell supernatants by ELISA. A modest release of IL-1β was observed with as-grown quartz only at the highest dose, while a remarkable dose-dependent release was induced by fractured quartz.
*(ANOVA +Tukey's) *p< 0.05, and ***p< 0.001 vs control; ##p< 0.01, and ###p< 0.001 n-Qz-syn vs µ-Qz-syn-f*

**As-grown synthetic quartz crystals are non-membranolytic, do not induce cell toxicity in macrophages and an inflammatory response. Crystal fracturing triggers the onset of cell toxicity and inflammatory events**

## 3. Molecular determinants of cell tox

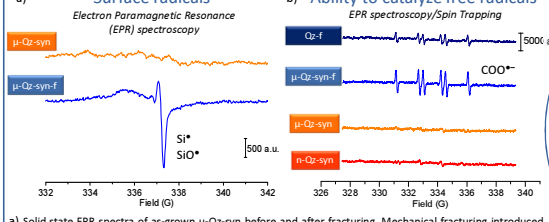
### Oxidative potential

a) Surface radicals

*Electron Paramagnetic Resonance (EPR) spectroscopy*

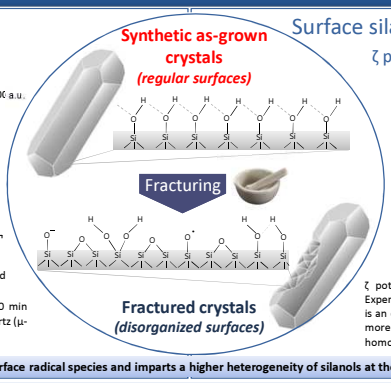
b) Ability to catalyze free radicals

*EPR spectroscopy/Spin Trapping*



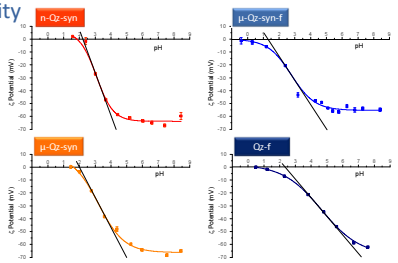

a) Solid state EPR spectra of as-grown µ-Qz-syn before and after fracturing. Mechanical fracturing introduced radical defects, absent on the pristine as-grown quartz crystal.

b) Carbon-centered free radicals generated in an aqueous suspension containing sodium formate after 60 min incubation with quartz as the result of the homolytic cleavage of a C-H bond in the formate ion. As-grown quartz (µ-Qz-syn, n-Qz-syn) showed no radical reactivity, fracturing (µ-Qz-syn-f) induced a strong, sustained reactivity.




### Surface silanol acidity

ζ potential




ζ potential *vs* pH of as-grown (n-Qz-syn, µ-Qz-syn) and fractured (µ-Qz-syn-f, Qz-f) quartz crystals. Experimental points are fitted with a non-linear curve. The slope of the tangent line at the inflection point is an estimate of the heterogeneity of surface silanol acidity. The steeper the slope of the tangent line the more homogeneous the acidic nature of the silanols. Synthetic as-grown quartz crystals showed a more homogeneous acidic silanol distribution than fractured crystals.

**Fracturing generates surface radical species and imparts a higher heterogeneity of silanols at the quartz surface**

## Conclusion and future perspectives

Present data support the hypothesis that the biological activity of quartz dust is not due to crystallinity *per se* but to the new faces produced during crystal fragmentation. Besides radical generation, fracturing upsets the long-range order of surface functionalities - silanols, silanolates, siloxanes - which disrupt membranes, induce an inflammatory response, and elicit cellular toxicity in macrophages. These cellular outcomes are associated to the pathogenicity of quartz. Further studies *in vivo* and on cellular markers of oxidative stress will be performed to confirm present results

### References


Donaldson K., and Borm P. (1998) *Ann. Occup. Hyg.* 42, 287-294; Fubini B. (1998) *Ann. Occup. Hyg.* 42, 521-530; IARC, International Agency for Research on Cancer (2012), 100C, 355-405; Pastero et al. (2016) *Cryst. Growth Des.* 16, 2394-2403;Turci et al. (2016) *Part. Fibre Toxicol.* 13:32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