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## Unveiling the variability of "quartz hazard" in light of recent toxicological findings

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# Unveiling the Variability of “Quartz Hazard” in the Light of Recent Toxicological Findings

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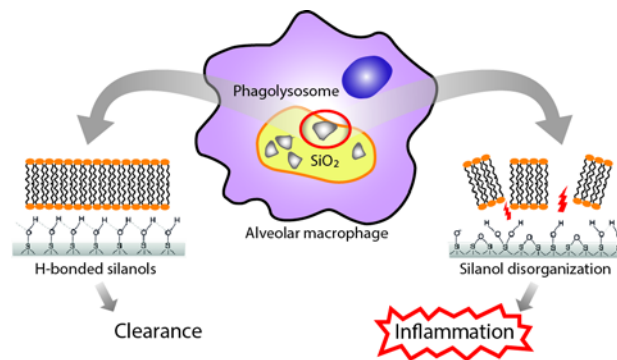
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ABSTRACT: The variability of quartz hazard stands as one of the most puzzling issues in particle toxicology, notwithstanding the fact that silicosis – the most ancient occupational disease – was the very topic from which the study of the toxicity of particulates developed. Over the years, other adverse effects of silica particles (i.e. lung cancer and autoimmune diseases) were detected and described. However, a few gaps are still present in the physico-chemical determinants and cellular pathways involved in the mechanisms of silica pathogenicity. In this “perspective”, we illustrate how pooling together studies in occupational health and nanotoxicology might fill such gaps, yielding a consistent picture of what imparts toxicity to a given silica. Recent investigations have shown that crystallinity is not implied in the pathogenic process of silica *per se*, while patches of disorganized silanols at the surface of both crystalline and amorphous particles can promote membrane damage and inflammation, a process at the origin of silica-related diseases. Introducing these new findings into the accepted multistep model of silica pathogenicity a picture is obtained of the chemical features of silica governing each cellular step in agreement with the outcomes of major previous studies. We ascribe the origin of the variability of silica hazard mainly to the distribution of various moieties at the particle surface, with silanols playing the major role. Toxicity turns out to be likely predictable by an *ad hoc* surface characterization. Tailored modifications of the surface can be envisaged to prepare safe materials or blunt toxicity in existing ones.

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## **1. The variability of quartz hazard**

### **1.1. Occurrence of silica-related diseases: circumstances and peculiarities**

Silicosis – the most ancient occupational disease – occurs with very variable severity depending on the source of crystalline silica, while no significant cases were reported following exposure to the most common sources of amorphous silica.<sup>1</sup> Lung cancer is associated to crystalline silica exposure, but does not occur in all circumstances of exposure to crystalline silica.<sup>2,3</sup> Such situation was summarized at the end of the century as “The quartz hazard: a variable entity”.<sup>4</sup> Silica is present worldwide in nature – rocks, sands, biogenic deposits, fly and volcanic ashes – and employed in a large variety of manufactures – glass, cement, ceramics, nanomedicine – yet diseases occur in a limited number of cases. The compound  $\text{SiO}_2$  cannot thus be considered pathogenic as such. The question is: how and when does  $\text{SiO}_2$  become a toxic agent?

Since ancient times crystallinity was considered to be the prerequisite feature for silica pathogenicity, in spite of some reports on non-pathogenic quartz dusts<sup>2,5,6</sup> and the general notion among mineralogists that quartz particles generated by grinding yield irregular distributions of crystallographic surfaces, expose conchoidal fractures,<sup>7,8</sup> and are mostly covered by an amorphous layer.<sup>9-12</sup> Cells and tissue would have indeed been in direct contact with amorphous surfaces. Observing the occurrence of major occupations associated to silica exposure and related disasters (Table 1), one feature emerges: in nearly all cases, crystalline silica (more often quartz, but also the other common polymorphs cristobalite and tridymite) was ground, crushed or abraded. This is also the case with the very recently-reported outbursts of silicosis due to denim sandblasting,<sup>13,14</sup> hydraulic fracturing of gas and oil wells,<sup>15</sup> and polishing artificial stones.<sup>16-18</sup>

**Table 1.** Major occupations associated to silica exposure and related disease outbreaks

Occupations	Industries	Associated outbreaks
Sandblasting	Construction, painting, shipbuilding, ironworking, denim and hydraulic fracturing	Sandblasters in oil fields, 1970-2000s, Texas and Mississippi (USA) <sup>19</sup> Denim-sandblasting in garment industry, 2000s, Turkey, <sup>13</sup> Bangladesh <sup>14</sup>
Mining	Mines (construction, metals and non-metals, diatomaceous earth, and coal mining).	Gold mines: 1900s, Delamar, Nevada (USA); 1940s, Australia; 1980-90s Minas Gerais, Brazil; <sup>20</sup> 2000s, South Africa, <sup>21</sup> and China <sup>22</sup> Hawks Nest tunnel, 1930s, West Virginia (USA) <sup>19</sup> Val Camonica construction site 1950s, Brescia (Italy)
Granite processing and mining	Quarries (granite)	Granite workers, 1970s, Vermont (USA) <sup>2</sup>
Grinding	Industrial sand, granite industry	
Ceramic production	Pottery, ceramics, sanitary ware, tiles, porcelain	Ceramic workers, 1970s-1980s, Italy, <sup>23</sup> USA, UK <sup>2</sup>
Glassmaking	Glass production	
Casting, blasting	Foundry	
Polishing, cutting	Construction, granite and artificial stone industry, jewelry and dental products	Outbreaks of silicosis among countertop workers, 2000s, Israel, <sup>18</sup> Spain, <sup>17</sup> Italy <sup>16</sup>
Drilling and fracking	Hydraulic fracturing	Hazard alert, 2016, USA <sup>15</sup>

Major pathologies caused by particulate matter – asbestos and silica – have been for long time associated merely to physical factors, e.g. fibrous shape and crystallinity, disregarding any role of surface reactivity. Only at the end of the past century, a large number of *in vivo* and *in vitro*

experimental studies have shed light on several surface physico-chemical features of the particles related to their adverse effects on cells and tissues.

### **1.2. The variability in cell responses to particulate silica**

Many studies showed that crystalline silica particles are cytotoxic, inflammogenic, trigger production of inflammatory, oxidants and growth factors from cells, can damage membranes etc.<sup>9,24</sup> However, when the same test was performed on a variety of silica samples either collected or prepared ad hoc, the extent and even the nature of the cellular response varied.<sup>5,25-34</sup> If the quartz particles were associated to other minerals or elements (such as aluminum, iron, carbon), effects were in some cases enhanced<sup>35-37</sup> or blunted in others.<sup>35,38-40</sup> Also, deposition of polymers or other substances on the particle surface modified (mostly inhibited) cellular responses.<sup>41-45</sup>

Remarkable differences were also found among various forms or preparation of amorphous silica. The interest in synthetic nanosilica – nearly all types of which amorphous – has grown exponentially in the last years due to their huge potential in medical, cosmetic, and food applications. Thus silica nanoparticles – for which a special abbreviation has been coined, SiNPs – have become one of the major issues of particle and nano- toxicology. A review appeared few years ago entitled “The nanosilica hazard: another variable entity”.<sup>46</sup> Further studies confirmed such variability,<sup>47-49</sup> and showed that some amorphous silica types elicited cell responses considered relevant to the accepted mechanism of toxicity of crystalline silica, and their effects were sometimes comparable to or even more pronounced than those of crystalline silica particles.<sup>50,51</sup>

### **1.3. Silica itself as a variable entity: the physico-chemical basis of its intrinsic variability**



Silica, the most ancient material used by humankind – flint stones mainly consist of silica –, has a simple chemical formula,  $\text{SiO}_2$ . Opposite to other oxides, e.g.  $\text{ZnO}$ ,  $\text{NiO}$ ,  $\text{TiO}_2$ , silica is always stoichiometric. Yet silica occurs in a wide variety of polymorphs and forms, which give rise to very different materials.<sup>52,53</sup> One should therefore refer to the plural term “*silicas*”, as silica is not a single entity, or just use silica as a plural noun. Nothing similar happens with any other oxide. The reason for this peculiarity mainly stems from the specific nature of the silicon-oxygen bond: a covalent *sigma* bond, with polar character and with a large degree of flexibility of the Si–O–Si angle that connects two tetrahedra.<sup>52</sup>

*Flexibility of the Si–O–Si bond and the presence of a large variety of silica.* The silicon-oxygen tetrahedron  $[\text{SiO}_4]$  is the basic unit of all silica forms, with the only exception of stishovite where silicon is hexa-coordinated. In the tetrahedron each silicon atom is surrounded by four oxygen atoms and each oxygen atom is shared by two tetrahedra. While the  $[\text{SiO}_4]$  unit is rigid, the Si–O–Si angle that connects two tetrahedra can easily change upon temperature, pressure or geometrical constraints. Due to this high flexibility, a large number of silica-based materials are found in nature or may be prepared industrially for different purposes, from dense crystalline and amorphous structures (e.g. quartz or glasses) to porous light systems (e.g. aerogels, zeolites and microporous/mesoporous materials). Most common natural sources of silica are of mineral or biogenic (i.e. silica materials produced by living matter) origin. Both natural and synthetic sources of silica may exhibit amorphous or crystalline forms.<sup>52</sup>

Crystalline silica is characterized by regular arrangements of the tetrahedra, and exists in a relatively large number of natural polymorphs depending on the different orientation and position of the tetrahedra,<sup>2</sup> a direct consequence of the flexibility of the bond joining the tetrahedra. Alpha-quartz is the thermodynamically stable polymorph in ambient conditions, thus

the most present in nature (12% of the earth crust). The other polymorphs, tridymite, cristobalite, coesite and stishovite may be found in a metastable state at room temperature and atmospheric pressure over extremely long periods of time<sup>2</sup> (for details on stability and metastability see the phase diagram).<sup>54</sup> When heated above ca. 1700 °C silica melts. The cooling down of the melt gives rise to vitreous silica, made up by a disordered network of [SiO<sub>4</sub>] tetrahedra. Thus vitreous silica is amorphous, albeit very similar to quartz.<sup>50</sup> Synthetic crystalline silica particles are not very common, but examples are some high silica forms (i.e. porosils) similar to the most common aluminosilicates (zeolites)<sup>55</sup> or cultured quartz crystals.<sup>56,57</sup>

In amorphous structures the [SiO<sub>4</sub>] units are linked in random networks and the orientation of the bonds lacks any periodicity, which yield disordered systems. Amorphous natural forms are either of mineral origin, such as hydrous silica (e.g. opal) and vitreous silica, or of biogenic origin. Two major biogenic silica sources are diatomaceous earth, formed by deposition of diatoms frustules, or crop plants such as sugar cane, rice and millet, which accumulate silica in their tissues to promote structural integrity and protect against pathogens and insects. Synthetic silica is generally produced in amorphous form, examples being pyrogenic and precipitated silica.

*Covalence and polarity give rise to the coexistence of several radical functionalities.* The intermediate nature of the Si–O bond between covalence and polarity explains why two types of cleavage are possible:<sup>9</sup> i) a *homolytic fracture* leaving an unpaired electron on both silicon and oxygen atoms (dangling bonds), which readily react with atmospheric components, mostly O<sub>2</sub>, forming oxygen-based surface radicals;<sup>58</sup> ii) a *heterolytic fracture* which, because of polarity, generates a positive charge on silicon and a negative one on oxygen, which may either

coordinate metal ions or attract smaller particles at the surface of bigger ones by electrostatic interactions.

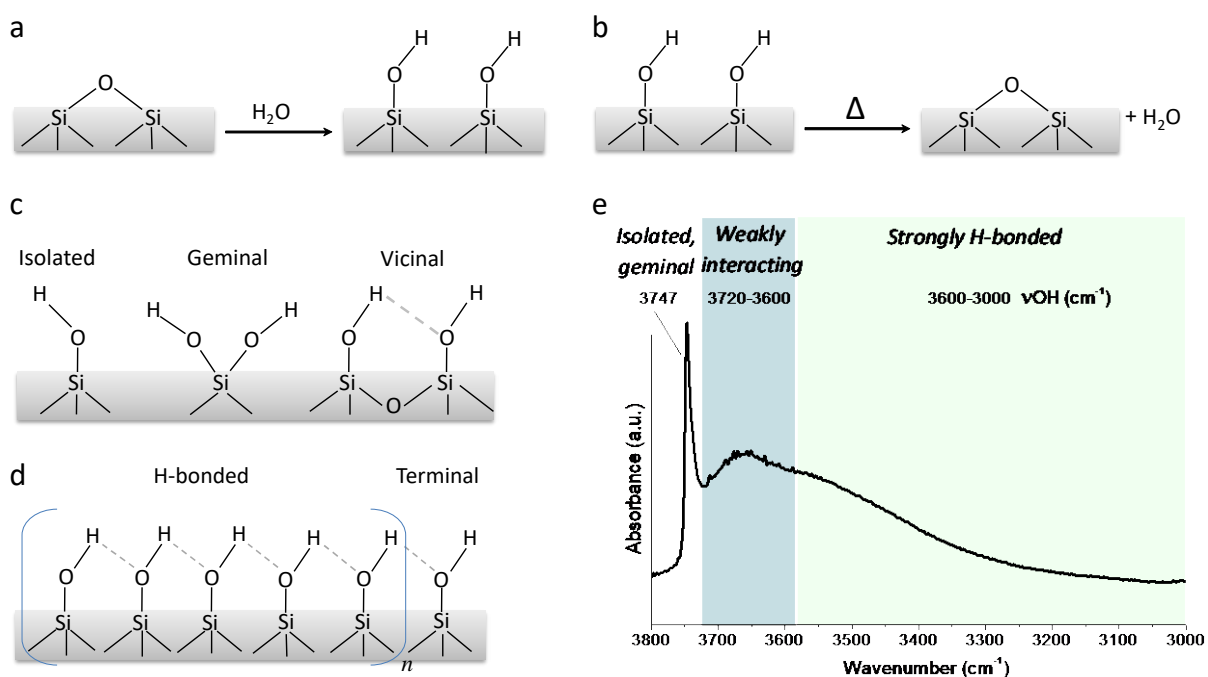
Interestingly, beside the gaseous environment, the chemical composition of the grinding chamber will also determine the surface topography and location of possible contaminants.<sup>36,59</sup> In very moist atmosphere water will assist saturation of broken bonds reducing radicals and generating silanols (see next chapter).<sup>60</sup>

*Polarity and interaction with water determine silanol population, surface acidity and H-bonding potential.* Any ideal termination of pure silica envisages arrays of Si–O–Si bonds, i.e. siloxane bridges, either ordered (crystalline) or disordered (amorphous). Because of the polarity of the Si–O bond, in the presence of water, siloxanes tend to dissociate the H<sub>2</sub>O molecule to form two silanols (–SiOH)<sup>61</sup> (Figure 1a). This process may readily occur upon exposure to atmospheric moisture with strained siloxane bridges or be extremely slow with regular ordered ones.

Conversely, silanols may condense into siloxanes upon heating via the reverse reaction (Figure 1b).<sup>62</sup> At the silica surface the condensation process may also form siloxane rings (SiO)<sub>n</sub>, from the highly strained two- and three- membered silicon rings – (SiO)<sub>2</sub> and (SiO)<sub>3</sub>, respectively – to larger unstrained ones.<sup>48,52</sup> A full surface hydration – i.e. complete reaction of siloxanes with water – mainly takes place when silica particles are synthesized by precipitation from an aqueous solution, while full conversion of silanols into siloxanes may be only attained at very high temperature.<sup>62</sup> Besides these two extreme situations, all other silica surfaces will be partially hydrated, in a metastable state towards water molecules. Opposite to what happens with metal oxides, silanols are acidic moieties with a potential for H-bonding, the strength of which depends upon their location at the surface.

Any silica surface is characterized by different types of  $\text{-SiOH}$  groups and by different H-bonding patterns resulting from mutual interactions among them.<sup>52</sup> The various “families” of silanols that coexist on most silica surfaces, as illustrated in Figure 1c, are: *isolated*, when the distance to the closest  $\text{-SiOH}$  groups is such (more than  $\text{ca. } 3.3 \text{ \AA}$ ) that they are unable to establish mutual interactions; *geminals*, two hydroxyl groups ( $\text{-OH}$ ) linked to the same silicon atom that also cannot be involved in mutual H-bonding because of the  $\text{-OH}$  orientations; *vicinals* belonging to tetrahedra that share a common oxygen vertex. Even if separated by less than  $3 \text{ \AA}$ , vicinal silanols usually do not establish mutual H-bonds or, as consequence of local geometrical constraints, are involved in weakly H-bonding. The isolated and geminal silanol groups are then free to establish H-bond interactions with external molecules as H-bond donor and acceptor sites but only if in the right position, as the nature of H-bonding is strongly directional. Two silanols that do not belong to directly connected tetrahedra (as the vicinal ones) or the same tetrahedra (as the geminal ones) but nonetheless are closer than  $3.3 \text{ \AA}$  will establish H-bonding, and are called *interacting* or *H-bonded* (Figure 1d). According to Rimola et al.,<sup>52</sup> the optimum  $\text{O}\cdots\text{O}$  distance between the two  $\text{-OH}$  involved in a H-bond lies between  $\text{ca. } 2.5$  and  $2.8 \text{ \AA}$ . The H-bond patterns strongly depend on the disposition and on the density of the  $\text{-SiOH}$  groups at the surface.

Presence of these silanol patterns on a silica surface give rise to different spectroscopic manifestation due to  $\text{-OH}$  stretching ( $\nu\text{OH}$ )<sup>63</sup> as shown in Figure 1e where a typical FT-IR spectrum in the hydroxyl spectral region ( $3800\text{-}3000 \text{ cm}^{-1}$ ) of a pyrogenic silica is taken as an example.<sup>26</sup> The narrow peak at  $3747 \text{ cm}^{-1}$  is associated to isolated or geminal silanols (distinguishable only by NMR spectroscopy),<sup>52</sup> the band at  $3720\text{-}3600 \text{ cm}^{-1}$  to  $\text{-SiOH}$  interacting via van der Waals or weak H-bonding, the broad band at  $3600\text{-}3000 \text{ cm}^{-1}$  to  $\text{-SiOH}$  interacting via strong H-bonding.<sup>64</sup>



**Figure 1.** Silanol groups and patterns on a silica surface. (a) Formation of silanol groups from siloxane opening in presence of water. (b) Condensation of silanols into siloxanes upon heating. (c) Different silanol groups and (d) H-bonding patterns on a silica surface. (e) FT-IR spectrum in the hydroxyl spectral region ( $3800\text{--}3000\text{ cm}^{-1}$ ) of a pyrogenic silica. The narrow peak at  $3747\text{ cm}^{-1}$  is associated to isolated or geminal silanols, the band at  $3720\text{--}3600\text{ cm}^{-1}$  to silanol interacting via van der Waals or weak H-bonding, the broad band at  $3600\text{--}3000\text{ cm}^{-1}$  to silanols interacting via strong H-bonding.

Because of the complex kinetics governing surface hydration-dehydration, the surface distribution of siloxanes and of the various silanol families varies from one to the other source of silica particles and may slowly evolve with time. Siloxanes and isolated silanols impart hydrophobicity to the surface, while all other types of silanols cause hydrophilicity.<sup>62</sup> A highly

dehydrated surface is hydrophobic while a fully hydrated one is hydrophilic. Besides these two extremes any silica surface will bear hydrophilic and hydrophobic patches, the distribution of which will be characteristic of that specimen and its thermal history.

For more details, an extensive literature (both past or recent) on the chemistry of silica is available.<sup>52,53,65,66</sup>

*Why is silica unique?* The above description accounts for a variability of silica hazard, as there is now agreement (see below) that it is the surface of the particle which interacts with living matter and that several surface features – typically dangling bonds, free radical generating centers, silanols – are implicated at different stages in the pathogenic mechanism underlying silica health effects. Moreover, most of the surface functionalities may react with many of the impurities in contact with the surface – such as charges or silanols with metal ions – further enhancing surface heterogeneity, hence variability.<sup>67</sup> The answer to the question: *why silica is unique?*, is so straightforward. Silica is one of the few stable, non-ionic oxides always present in solid state in ambient conditions. A ionic oxide, particularly when in aqueous medium, will easily undergo surface reconstruction, healing of defects, release of metal ions, compensation of charges, yielding surface states much simpler than those of silica.

## **2. A brief overview on the approaches to the mechanism(s) of silica pathogenicity: from simple association between particle exposure and injury to a multistep pathological process governed by different physico-chemical features**

The search for the characteristics of crystalline silica particles causing adverse effects took place much time later than the detailed description of the diseases and of its anatomical features, e.g. the silicotic nodule. It has however to be mentioned that well-conducted pioneering studies in the 1950s already indicated a possible role of chemistry underlying the toxicity of silica.<sup>11,68-70</sup> Research then continued in search of one single physico-chemical property responsible for the toxic effects of silica. Some features of crystalline silica absent in amorphous ones appeared the best candidates.

### **2.1. Cells and mediators implicated**

In the mid-20th century it became clear that several cell types are involved in damage of the lung tissue in silicotics, including macrophages, granulocytes, epithelial cells, and fibroblasts. A mechanistic model implying macrophage activation and/or damage, release of several reactive oxygen and nitrogen species (ROS and RNS, respectively), pro-inflammatory and chemotactic mediators (e.g. cytokines, chemokines, growth factors, alarmins, arachidonic acid derivatives, enzymes), followed by fibroblast proliferation was proposed and progressively implemented with new findings.<sup>4,71,72</sup> Inflammation was considered a crucial step not only in silicosis but also in silica-related bronchogenic carcinoma.<sup>3,73</sup> Yet the physico-chemical features of crystalline silica responsible for all the above adverse outcomes were still obscure. When the need to unveil the basic reasons behind toxicity was required for prevention and therapy, attention was finally focused on the physical-chemical characteristics of the particles.

### **2.2. The central role of the particle surface**

Much time later the particle surface was recognized as the chemical entity in direct dialog with cells and tissues and particularly with cell membranes. Accordingly, the majority of the antidote used for silicosis were substances that would modify or cover the particle surface. Surface modifications such as etching with HF, aluminum contamination, polymer deposition, impurities, appeared indeed to modulate cellular responses.<sup>33,38,41,42,74-77</sup> The crucial role of the particle surface in evoking toxic effects was then accepted. Research was, however, still focused on one single feature that would have explained silica toxicity. When a limited number of crystalline silica dusts or of chemically modified crystalline silica particles were tested, several surface properties appeared to parallel some of the adverse cellular responses or even toxicity *in vivo*: the type of polymorph,<sup>6,31,33,69,71,78</sup> particle shape and size,<sup>28,79</sup> free radical release,<sup>29,41,58,80-82</sup> the degree of hydrophilicity.<sup>30,83</sup> But when the association of a given feature to toxicity was checked across different studies, the hypothesis did not hold anymore. Sticking to one single feature is probably the cause which limited the physico-chemical understanding of the molecular mechanisms until recent times.

In experiments on sets of crystalline silica samples with a wide range of toxicity, when different cellular endpoints were investigated it became clear that most of the biological responses relevant to the toxic activity were independent one from the other, e.g. the most fibrogenic silica was not the most genotoxic,<sup>5,25</sup> membranolysis was not directly related to cytotoxicity nor was cellular oxidative stress.<sup>33,34,84</sup> Clearly, the mechanism of toxicity of crystalline silica was not only complicated by the occurrence of several biochemical events contributing to pathogenicity, but also by the various silica features associated to each one of the events. Several physico-chemical features, which are reported in Table 2, have been indicated as determinants for some steps of the pathogenic mechanism of silica particles.



**Table 2.** The bulk and surface physico-chemical features of silica determining the variability of its toxic effects

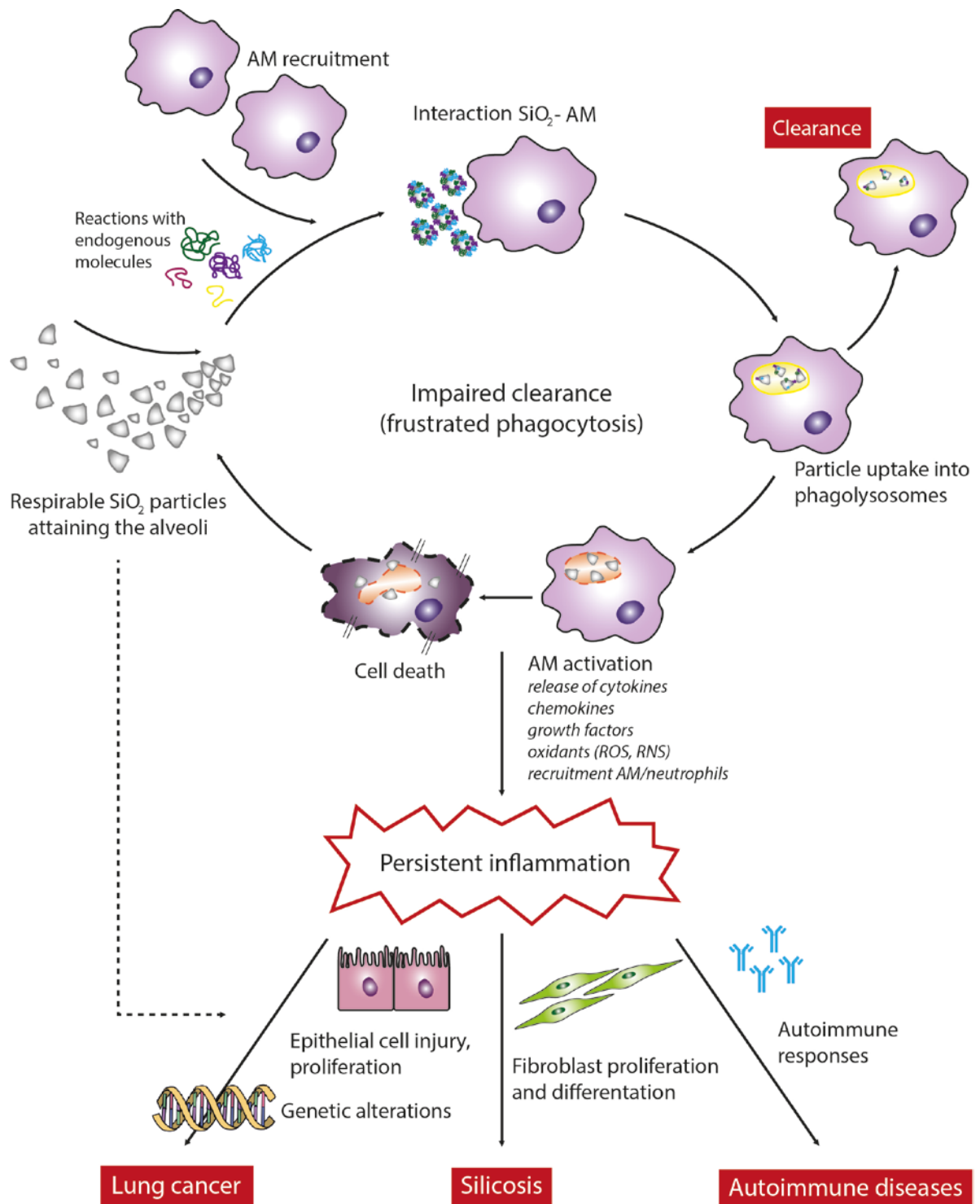
	Type	Origin and details
Bulk features	Crystal structure	Ordered (crystalline silica) or irregular (amorphous silica) arrangement of the [SiO <sub>4</sub> ] tetrahedra.
		Different orientations and positions of the ordered [SiO <sub>4</sub> ] tetrahedra (crystalline polymorphs).
	Size and morphology	Dependent on particle origin. Particles obtained by grinding processes usually exhibit irregular surfaces, acute spikes, and edges. Their size is usually heterogeneous, from few microns to nano-scale fragments. Particles obtained by combustion or precipitation show smooth and roundish surfaces of almost the same size, from few micron to nanometers.
Surface features	Solid-based radicals and ROS	Solid-based radicals associated to fracturing: silyl radical ( $-\text{Si}^\bullet$ ) (E' center) siloxyl radical ( $-\text{SiO}^\bullet$ )
		Surface ROS associated to surface reconstruction of solid-based radicals reacting with atmospheric components: hydroxyl radical ( $\text{HO}^\bullet$ ) superoxide radical ( $-\text{Si}^+ \text{O}_2^\bullet$ ) peroxy radical ( $-\text{SiO}_2^\bullet$ )
	Silanol/siloxane groups	Associated to surface reconstruction and hydration/dehydration processes: isolated silanol ( $-\text{SiOH}$ ) geminal silanol ( $-\text{Si}(\text{OH})_2$ ) vicinal silanols (from SiO <sub>4</sub> sharing a common oxygen vertex) H-bonded silanols symmetric siloxane bridge ( $\text{Si}-\text{O}-\text{Si}$ ) distorted siloxane bridge ( $\text{Si}-\text{O}-\text{Si}$ ) siloxane rings ( $(\text{SiO})_n$ (n= 2-6)
	Charges	Associated to fracturing: silyl cation ( $-\text{Si}^+$ ) siloxyl anion ( $-\text{SiO}^-$ )
		Associated to silanol deprotonation in aqueous suspension: silanolate ( $-\text{SiO}^-$ )
	Presence of metal ions and impurities	Substituting for Si in the [SiO <sub>4</sub> ] framework: $\text{Al}^{3+}$ , $\text{Fe}^{3+}$
		Adsorbed on silica surface: $\text{Al}^{3+}$ , $\text{Fe}^{2+}$ , $\text{Fe}^{3+}$ , $\text{Cu}^{2+}$ , $\text{Na}^+$ , $\text{K}^+$ , $\text{Mg}^{2+}$ , $\text{Ca}^{2+}$ , C

### **2.3. Subsequent steps of the mechanism of silica toxicity as modulated by different surface properties**

The following scenario leading to silica pathogenicity is thus set up – still holding a few gaps – as illustrated in Figure 2. The inhaled silica particle attains the alveolar space where proteins and surfactants tend to cover the particle surface,<sup>9</sup> while endogenous antioxidants might react with the particle surface and be inactivated, a process involving ascorbic acid and glutathione which may enhance the oxidative stress caused by cell and particle-derived ROS.<sup>85,86</sup> The particle is engulfed by alveolar macrophages (AMs) into a phagosome that fuses with lysosomes to form a phagolysosome. AMs will then attempt to digest or clear the particle out of the lung.<sup>4,67</sup> Clearance will be successful only with some/few dust specimens, not yet well defined, while AM will be mostly activated, and eventually the phagolysosome membrane will be damaged inducing pro-inflammatory and cell death pathways.<sup>51,87-92</sup> The particle itself and all of AM products will consequently be deposited onto the alveolar epithelium. New macrophages and neutrophils will be then recruited, a circular process causing a persistent inflammation which will proceed as long as the particle remains in the alveolar space. The sustained inflammatory response, accompanied by fibroblast stimulation and growth, abnormal collagen synthesis, and fibrosis, will cause a cascade of events – oxidants release, epithelial cell injury, genetic damage – ending up with bronchogenic carcinoma.<sup>3,24,73,93</sup> An additional pathway whereby release of oxidants and direct injury of the particles on the epithelial cells is also hypothesized,<sup>43,94,95</sup> which would add further damage.

Clearly, the mechanism proposed for silica pathogenicity is made up by various subsequent steps. This fairly accounts for the variability of quartz hazard, the overall toxic potential being the result of the contribute of the different characteristics involved in each step and their

abundance at the particle surface. The association of a given physico-chemical property to each of the steps, however, is far from being completed.



**Figure 2.** Proposed mechanism of toxicity of a silica particle attaining the alveolar space. The particle may directly stress the epithelium (dotted line), while the major pathway – following interaction with endogenous matter (antioxidant inactivation and surface coverage by proteins) – involves recruitment of alveolar macrophages (AMs) → phagocytosis by AMs → clearance or AM activation (release of pro-inflammatory, pro-fibrotic mediators and chemotactic factors) following damage to the phagolysosome membrane → eventually cell death → release of particles and AM products → recruitment of new AMs and neutrophils. A continuous ingestion-re-ingestion cycle, AM activation and death is established. The prolonged recruitment/activation of AM and neutrophils causes a persistent inflammation which may evoke: i) epithelial cell injury and proliferation, genetic alterations, and ultimately lung cancer; ii) fibroblast proliferation and differentiation, collagen production and finally silicosis; iii) autoimmune responses ending in autoimmune diseases (such as systemic lupus erythematosus, systemic sclerosis, rheumatoid arthritis, vasculitis, and chronic renal disease).

### **3. Filling the gaps in the association between particle surface features and the single steps in the cascade of events causing silica pathogenicity**

Gaps to be filled require, on the one hand, to consider in a new perspective past paradigms, and, on the other hand, to carry out studies where the interaction particle - living matter in each step contributing to silica pathogenicity is simplified as much as possible.

#### **3.1. Objections to the paradigm of crystallinity in silica-related diseases**

The crystal structure is seldom in direct contact with cells and tissues. Some recent studies even suggest that crystallinity *per se* is not a cause of pathogenicity.<sup>96</sup> Main points to consider are:

- not all crystalline silica particles are pathogenic
- ground vitreous silica behaves like a quartz dust
- some amorphous silica particles exert toxic effects on cells similarly to crystalline ones
- the crystalline order of the bulk seldom shows up at the particle surface

*Not all crystalline silica particles are pathogenic.* It is known that not all crystalline silica polymorphs are toxic. Indeed, the dense polymorphs coesite and stishovite were found inert in toxicological studies if compared to quartz, cristobalite, and tridymite.<sup>6,28,71,97</sup> Interestingly, physico-chemical investigations have shown that stishovite is characterized by a more densely-packed structure of the –OH groups, which involves stronger H-bonding interactions among silanol groups, and stishovite does not generate surface radicals even when ground.<sup>98</sup> Moreover, some quartz dusts were occasionally found inactive in experimental studies.<sup>3,5,25,32</sup>

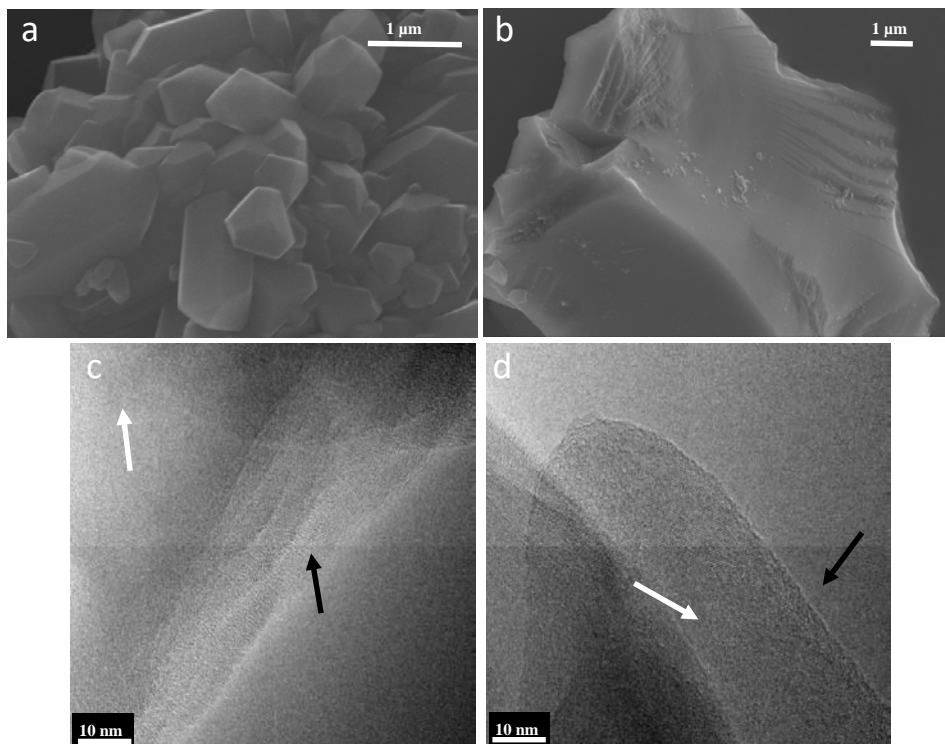
*Ground vitreous silica behaves like a quartz dust.* Vitreous silica (or silica glass) is obtained by rapid solidification of the melt and is fully amorphous. Opposite to amorphous forms obtained

by combustion or precipitation, vitreous silica is present in particulate form only by fracturing, grinding, or abrading. Vitreous silica particles show the same micromorphology (irregular surfaces and pointed edges) and reactivity of quartz in acellular and cellular tests.<sup>26,27,50</sup> These include potential to generate free radicals in simulated biological fluid, hemolytic activity, and cytotoxic and pro-inflammatory activity in alveolar macrophages. Significantly, several cases of silicosis and lung cancer have been reported in the past among workers who use quartz as the primary raw material but are mostly exposed to vitreous silica particles in glass factories.<sup>99-101</sup>

*Amorphous silica particles may exert toxic effects on cells.* The potential pathogenicity of amorphous silica is still controversial, but some amorphous silica powders induce on cells some of the effects which are determinant in crystalline silica pathogenicity.<sup>46-49,84,102-105</sup> When prepared via pyrolysis (e.g. pyrogenic or fumed silica), amorphous silica is much more active in vitro than when obtained via wet processes (e.g. precipitated, colloidal, and mesoporous silica).<sup>106</sup> Pyrogenic silica is more membranolytic,<sup>26,48</sup> cause stronger oxidative stress and cytotoxic and pro-inflammatory effects both in macrophages,<sup>47-49,51,102</sup> and epithelial cells<sup>48</sup> than precipitated one. Several hypothesis have been proposed to explain these results such as a different degree of hydrophilicity/hydrophobicity,<sup>49</sup> and presence of strained siloxane rings on pyrogenic silica.<sup>48</sup> Inhalation studies regarding pyrogenic silica reported remarkable expression of lung inflammatory and histopathological markers, which were much milder or even absent with precipitated silica.<sup>48,107,108</sup> It has to be stressed, however, that in vivo both pyrogenic and precipitated silica – opposite to crystalline dusts – cause only transient and reversible lung inflammation with no progressive lung fibrosis.<sup>3,46,107-111</sup> This notwithstanding the difference reported among amorphous silica types may be a clue to discover the nature of the surface sites involved in crystalline silica pathogenicity.

*The crystal order of the bulk seldom shows up at the particle surface.* Native uncontaminated quartz particles made up of perfect crystal faces as the ones showed in Figure 3a very rarely are in direct contact with living matter, an aspect highly underestimated by toxicologists. Workers are mostly exposed to crystalline silica dusts generated by grinding/abrading macroscopic quartz crystals of mineral origin. In quartz – opposite to brittle solids such as asbestos – crystallographic planes of weakness (i.e. cleavage planes) are absent. Thus breakage causes a curved irregular fracture described as *conchoidal*, which does not follow a defined crystal plane, but crosses a variety of crystal planes.<sup>7,8,112</sup> The resulting exposed surface is characterized by a concatenation of numerous microscopic patches of single crystallographic arrangements, which likely will yield, upon contact with atmospheric moisture, a randomly distribution of silanols and siloxanes, similarly to what happens with amorphous materials.<sup>52,112</sup> The perturbed external amorphous layer (known as Beilby layer), which often covers quartz particles, stems from such process.<sup>10-12,113</sup> As a consequence the surface of a crystalline silica particle with whom cells interact is not a perfect and regular crystalline face, but rather an almost amorphous and irregular one. A scanning electron microscopy (SEM) image showing conchoidal fractures on a microscopic quartz particle obtained by ball milling of larger crystals is reported in Figure 3b. Transmission electron microscopy (TEM) images of a pure quartz dust prepared by milling (Figure 3c and d) show that the crystal lattice is only evident in the bulk of the quartz crystal, while a non-ordered thickness and displaced fractures are noticed at the edges.

*Silica pathogenicity is mostly confined to crystalline particles but many of the surface features known to be implied in crystalline silica toxicity are also shared by amorphous silica particles.*



**Figure 3.** Scanning electron microscopy (SEM) image of pure quartz particles laboratory-grown via hydrothermal synthesis (a); SEM (b) and transmission electron microscopy (TEM) (c, d) images of quartz particles obtained by ball milling of large quartz crystals. Synthetic quartz particles show intact, flat and smooth crystal surfaces (a), while conchoidal fractures are evident on milled particles (b, c). For milled particles, crystal structure (diffraction fringes, white arrows) is clear only in the bulk, while disorganization of the surface lattice and an amorphous layer (black arrows) are evident at the edges.

### 3.2. Simplified systems to build up a comprehensive model

As already stressed, a direct association between particle physico-chemical features and the corresponding steps in the pathogenicity process is not straightforward because of both the extreme variability of the surface states of silica compounds and the complexity of cellular



models. To learn more, the adoption of simplified yet scientifically sound and reproducible models of the interaction between particles and biological systems is required.<sup>114</sup>

*Simplified solids.* A perfect crystal interacting with a membrane is the most simplified approach to evidence the role of the crystal structure. If synthesized in respirable size, such crystal can offer to cells regular and ordered crystal planes and interacting silanol arrays in the absence of radical-generating centers, thus providing information on the role of sole crystallinity in the biological responses elicited.<sup>57,96</sup>

*Simplified biological systems.* Modeling of tissues and organelles have to be associated to the solid model particles so to evidence at the molecular level the nature and extent of interactions taking place. The cell membrane is the first site of interaction of the particle with cells. Since early toxicological studies the role of membrane damage in the toxicity mechanism of silica was considered.<sup>115-117</sup> The outer plasma membrane and the inner phagolysosome membrane are the possible membrane target of silica particles. To probe membranolytic effects of toxic particulates, red blood cells (RBCs) have traditionally been employed as convenient model of cell membranes that can release measurable hemoglobin following membrane perturbation and lysis. RBCs play no part in the pathogenesis of silicosis or lung cancer induced by silica, but already in the early 1960s the rupture of such simple membrane was considered a measure of the cytotoxicity of mineral particles.<sup>77,118-120</sup> RBC lysis was even proposed to predict the fibrogenicity of industrial dusts.<sup>121</sup> Far from being connected to fibrogenicity, the hemolytic activity turned out to be one of the best predictive tests of the inflammatory response by silica and other inorganic particles.<sup>32,41,122,123</sup> Artificial biomimetic membrane models and computer simulations may provide further mechanistic insights to confirm the results obtained with natural model of cell membranes.<sup>124-127</sup>

*The search of the surface moieties imparting to silica particles the potential to disrupt membranes appears therefore as one of the best candidates for clarifying the association between surface properties and inflammation.*

### **3.3. A new Adverse Outcome Pathway (AOP) for silica inflammogenicity**

*How the Molecular Initiating Event (MIE) may be figured out.* We have recently shown that synthetic quartz crystals grown in respirable size and with intact crystalline surfaces do not elicit any of the usual reactivity in a series of acellular and cellular assays relevant for the toxicity of crystalline silica.<sup>96</sup> Instead, when the same synthetic quartz was fractured, cytotoxicity and membranolysis were induced to a similar extent as one reference pathogenic quartz. These findings imply, on the one hand, that crystallinity *per se* is not the key factor in the toxic effects of silica particles, and, on the other hand, that, only after fracturing a regular quartz particle becomes toxic. Besides formation of surface radical species, fracturing causes loss of the long-range order of the non-radical surface moieties, i.e. silanols, silanolates, and siloxanes, formed as a consequence of the reaction of the fractured surface with atmospheric components. The surface reconstruction yields mixed hydrophilic and hydrophobic patches and disordered silanol arrays similar to the irregular surface of some types of amorphous silica<sup>52,112</sup> reported as highly membranolytic,<sup>26,48</sup> so that also these irregular surfaces created on perfect quartz crystals may interact strongly with both biological and artificial membranes inducing lysis.<sup>96</sup>

The above findings are consistent with previous studies reporting membranolysis (measured on RBCs or lipid vesicles) as poorly associated to radical chemistry, but mostly related to the concentration of some silanol groups, either isolated<sup>128</sup> or geminal.<sup>112</sup> Considering a wide set of silica particles – both crystalline and amorphous – we recently found out that membranolysis is not merely related to silanol density or to a specific group of silanols, but rather to a peculiar

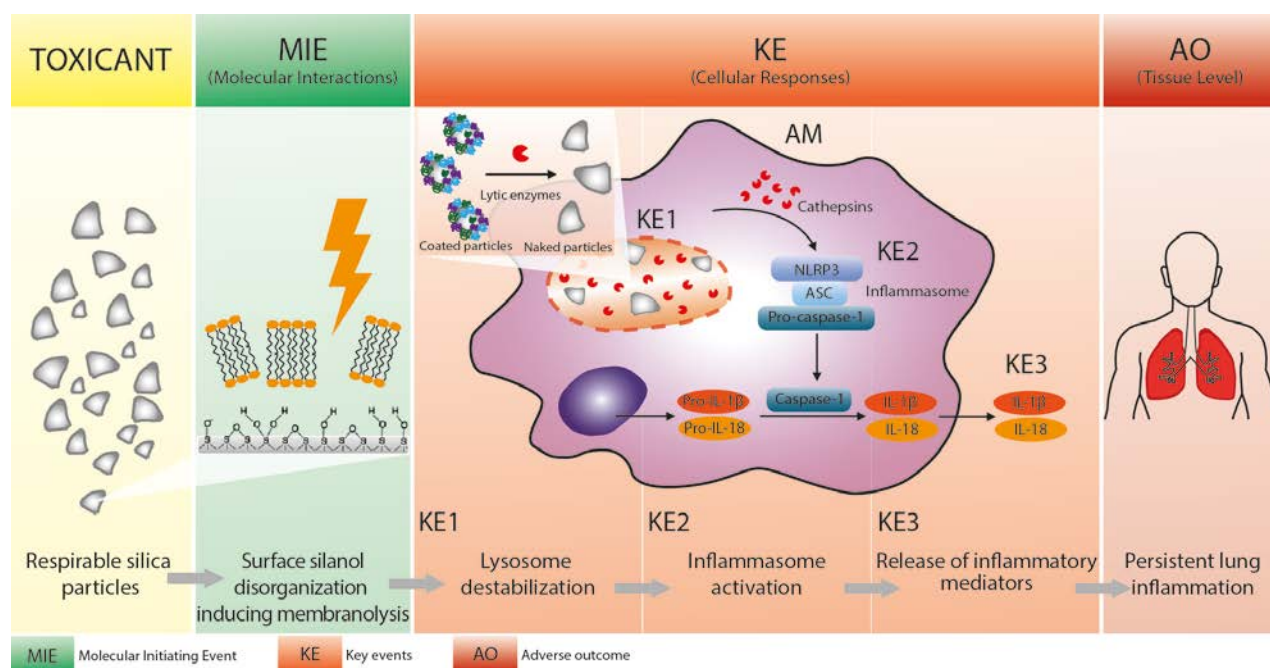
pattern of silanols, silanolates, and siloxanes.<sup>26</sup> We speculate here – also on the ground of data about to be published –<sup>129</sup> that silica surfaces characterized by a large silanol disorganization – imparted by fracturing, presence of metal impurities, physical treatments – are the most membranolytic ones.

*How the Key Events (KE) may be associated.* Hemolysis has been recently reported as one of the best predictive tests of *in vivo* inflammation following particle exposure,<sup>122,123</sup> including quartz,<sup>32,41</sup> but no explanation was proposed so far on the reasons of this correlation.

Recent studies have shown that silica particles induce the release of pro-inflammatory cytokines (e.g. IL-1 $\beta$ ) from different cell types via a membranolytic mechanism involving phagolysosomal membrane destabilization.<sup>89,90,92,130</sup> Rupture of the lysosome membrane following particle phagocytosis by AMs was reported since early studies on toxic particulate but acquired a clearer significance only at the beginning of the 21st century after the discovery of the inflammasome machinery as the triggering factor of the inflammatory response induced by pathogenic particles and fibers, including silica.<sup>90,131,132</sup> Indeed, one of the upstream biochemical mechanisms governing the silica-mediated activation of the NLRP3 inflammasome entails phagolysosomal damage and release of the lysosomal content into the cytosol. When activated, NLRP3 inflammasome can induce the proteolytic enzyme caspase-1 which is crucial in initiating cell death pathways and promoting secretion of pro-inflammatory cytokines (e.g. IL-1 $\beta$  and IL-8).<sup>89,91</sup> To understand the biochemical basis underlying the relationship between the hemolytic activity of silica and inflammation, we investigated the inflammasome-mediated pro-inflammatory response of a panel of silica samples selected for their different RBC lytic activity.<sup>27</sup> A clear cut correlation was evidenced between the hemolytic activity of the set of quartz particles and the release of IL-1 $\beta$  from macrophages following phagocytosis,

phagolysosome permeabilization and inflammasome activation. Consequently, we may infer that what is relevant for RBC membranolysis is also implicated in phagolysosome labilization, thus mediating inflammasome activation.

*A new proposal of AOP for silica inflammogenicity.* The AOP<sup>133</sup> proposed in Figure 4 stems from the association between recent physico-chemical and biochemical results suggesting that silanol disorganization is at the origin of the mechanism triggering the inflammatory reaction induced by silica particles.



**Figure 4.** Proposed Adverse Outcome Pathway (AOP) for silica inflammogenicity. Respirable silica particles deposited in the alveolar space (Toxicant), if in the membranolytic configuration characterized by silanol disorganization (Molecular Initiating Event, MIE), can disrupt the phagolysosome membrane (Key Event, KE1) when engulfed by alveolar macrophages (AMs). Within the phagolysosome, the particle surface coating due to adsorbed proteins and surfactants of the lung lining layer is removed by lysosomal enzymes. The unmasked silica surface can then

react with the inner phagolysosome membrane causing damage (inset to KE1). Release of the phagolysosome content into the cytosol, such as the proteases cathepsins B and S, triggers the activation of the inflammasome machinery (KE2) which leads to activation of the proteolytic enzyme Caspase-1 and release of active pro-inflammatory cytokines (i.e. IL-1 $\beta$  and IL-18) (KE3). This process induces and sustains the inflammatory response in the lungs (Adverse Outcome, AO).

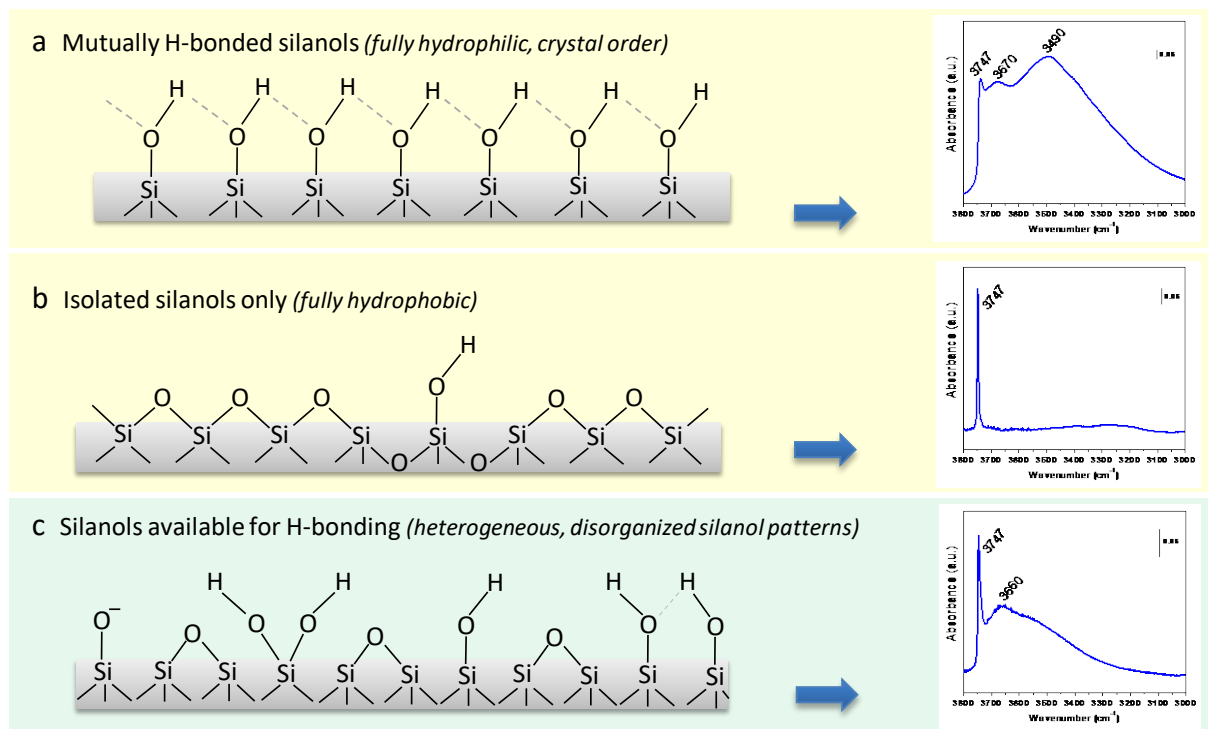
For the first time in the study of silica toxicity, the heterogeneity of surface functionalities is proposed as the key factor in the molecular initiating event (MIE) leading to the inflammatory response in the lungs (Adverse Outcome, AO) through a number of key events. The events recognized essential are cell membrane damage associated to the destabilization of the phagolysosomal membrane in alveolar macrophages and release of the phagolysosome content into the cytosol (Key Event 1, KE1). This latter event triggers the activation of the inflammasome machinery (KE2) which leads to the release of active pro-inflammatory cytokines (i.e. IL-1 $\beta$  and IL-18) (KE3), ultimately inducing and sustaining the inflammatory response in the lungs. Some studies further reports that the inflammasome machinery may ultimately affect fibroblast activation and proliferation,<sup>134</sup> thus contributing to the development of fibrosis and silicotic nodules.

Surface heterogeneity of oxides is a well-established concept in adsorption and catalysis<sup>61,135</sup> but less so in particle toxicology. As mentioned before, this is true not only for amorphous silica. Even crystalline silica, with a regular structure of the bulk, exhibits a lower organization at the fractured surface<sup>135,136</sup> where an amorphous layer is progressively formed during mechanical comminution of quartz crystals.<sup>10,67,113</sup> Opposite to metal oxides, the –SiOH functionalities in

silica never release hydroxyl anions but are strong H-bond donor and acceptor sites which may bind other molecules.<sup>9,52,115,118</sup>

We propose here that, when most silanols are mutually H-bonded in long-range arrays – on real crystal planes and highly hydroxylated surfaces – the silica surface is unreactive towards membranes (Figure 5a). The same happens on highly dehydrated surfaces where few isolated silanols are present on a surface (Figure 5b) mostly characterized by siloxanes. In both these situations strong H-bonds cannot be established with external molecules. In the first case because the long chains of mutually H-bonding silanols are stabilized by delocalized charges and it would be energy demanding to interrupt bonding among silanols so to establish new ones.<sup>137</sup> A different scenario is given by the intermediate situation in which a variety of more or less free silanols (e.g. isolated, geminals, weakly-interacting silanols, terminals) provide a distribution of strong H-bonding points on the surface, each one oriented in a well-defined direction and able to interact with external molecules (Figure 5c). Under such circumstances, some membrane components may strongly interact with particle surface, such as phosphate ester groups of phospholipids, secondary amide groups, nitrogen/oxygen in an amide of membrane proteins.<sup>124,126</sup>

Such a view is supported by some computational studies revealing that the energy of adsorption via H-bonding of small molecules like H<sub>2</sub>O and NH<sub>3</sub> anti-correlates with the density of –SiOH groups at the surface.<sup>137</sup> The strength of the interaction between the silica surface and biomolecules thus depends on the presence of available H-bonding silanols, not mutually interacting. Electrostatic interactions, localized charges on the particles, and charged moieties on the surface of membranes may also be superposed to the network of H-bonds.<sup>77,120,125,138</sup>



**Figure 5.** Chemical bases of the proposed MIE for silica consisting in silanol patterns which can be measured by IR spectroscopy. Silica surfaces characterized by (a) mutually H-bonded silanols – such as on real crystal planes and highly hydroxylated surfaces – or (b) few isolated silanols and siloxanes – such as on fully hydrophobic surfaces – are non-membranolytic. Silica surfaces characterized by silanols available for H-bonding with external molecules – such as in the case of heterogeneous and disorganized silanol patterns containing geminal, isolated, and weakly-interacting silanols – are membranolytic (c). In the right side of the figure, the FT-IR spectra in the hydroxyl spectral region ( $3800\text{--}3000\text{ cm}^{-1}$ ) of three silica particles representative of the three surface silanol patterns are represented. IR spectra recorded after outgassing at room temperature (RT) of: (a) a fully hydrophilic precipitated silica – showing predominance of strongly H-bonded silanols (broad band at  $3600\text{--}3000\text{ cm}^{-1}$ ), (b) a pyrogenic silica heated at  $800\text{ }^{\circ}\text{C}$  in vacuum – showing isolated/geminal silanols only (narrow peak at  $3747\text{ cm}^{-1}$ ), and (c) an as-produced

pyrogenic silica – exhibiting isolated/geminal silanols (narrow peak at 3747 cm<sup>-1</sup>), and weakly-interacting silanols (band at 3720-3600 cm<sup>-1</sup>).<sup>26</sup>

The AOP proposed for silica-induced lung inflammogenicity relies on two different coating or non-coating- mediated particle-membrane interactions. When in the bronchoalveolar region, the inhaled particle is coated by components of the lung lining fluid – formed by amino acids, proteins, lipids, etc. – and this adsorbed layer will mediate the interaction with the outer plasma membrane, reducing or inhibiting damage;<sup>139</sup> conversely, when the particle is engulfed by AM and internalized into a phagolysosome, the surface coating will be removed from the particle surface by lysosomal enzymes.<sup>87,114,140</sup> The unmasked silica surface can then react with the inner phagolysosome membrane causing membrane damage (Figure 4, insight) and consequently inflammation.

The disruption of phagolysosomal membrane leading to its permeabilization is not a unique phenomenon for silica particles. It has long been proposed for other particles, and most recently a unified theory for particle-induced inflammation which is based on lysosomal membrane permeabilization has been introduced.<sup>141</sup> Thus, investigations similar to those presented here for silica performed with other particles and fibers might elucidate similarities or differences in the mechanisms involved in their ability to induce permeabilization of phagolysosomal membranes.

*The sequence of events here proposed, that is: silanols available for H-bonding with external molecules → engulfment of coated particles by macrophages → strong interaction of the uncoated particle with the phagolysosomal membrane and rupture → macrophage activation → inflammatory response, is in line with the recent literature stressing acute and persistent inflammation as major cause leading to silicosis, autoimmune pathologies and lung cancer.<sup>2,3,73</sup>*



*The variability, which is intrinsic in silanol distribution on the silica surfaces, therefore determines a variability in the inflammatory response, thus in pathological outcomes.*

#### 4. Is the model proposed consistent with past research?

Earliest works considering chemical modifications of quartz<sup>33,142,143</sup> reported a variation in toxicity following treatments with HF or KOH. Both treatments do indeed modify the silanol distribution. Similarly, a rough correspondence can be noted between the evaluated toxicity and the expected abundance of reactive silanols in those studies where a different biological activity among silica specimens was reported.<sup>4-6,25-33,35-45</sup>

Here we would like to discuss the most obvious questions that our proposal may arise.

*Crystallinity, polymorphs and poorly active quartz.* The reason of the long term pathogenicity being confined to crystalline particles likely relies on the extremely low solubility,<sup>53</sup> hence high biopersistence of crystalline forms<sup>3</sup> and on free radical-generating centers, typically detected on quartz and cristobalite.<sup>58,144-147</sup>

The intrinsic differences in pathogenicity among the various polymorphs may be due to their density. The two most dense polymorphs – coesite and stishovite – are inert, which might be well ascribed to a dense packing of mutually interacting silanols. This was indeed the case on stishovite.<sup>98</sup> As to cristobalite, tridymite and quartz, the differences reported among them<sup>6,28,31,33,69,112</sup> are not larger than what reported among some sets of quartz dusts of different origin.<sup>5,25</sup> They are all potentially toxic. Note that tridymite was not classified by the International Agency for Research on Cancer (IARC) simply because of lack of data.

*How do silicosis antidotes act?* The major treatments which decrease or blunt silica toxicity are either metal ions, carbon, surface functionalization or coating. The most typical metal decreasing the adverse effects of quartz is aluminum in the form of aluminum salts,<sup>41-43,74,77,148</sup> as proposed long ago by Haldane,<sup>149</sup> or as clay/ kaolin<sup>150</sup> in direct contact with quartz. Aluminum ions may react with silanols, thus reducing their H-bonding potential. Also other ions may act

similarly. Iron is a double-edged sword because on the one hand, at low concentration, acts as free radical-generating center on quartz surfaces,<sup>4,151</sup> and, on the other hand, when in large amount, offers a masking effect of silanols and silanolates.<sup>29,39</sup>

Carbon in intimate contact with quartz was also shown to completely inhibit particle and cell-derived oxidative stress, and to reduce inflammatory responses and cytotoxicity in macrophages. None of these effects took place when the same experiments were carried out with mechanically mixed samples, suggesting that carbon acts not just as a radical quencher but because of its association to the quartz surface.<sup>152</sup>

Probably the most employed antidote against silicosis was the polymer PVPNO (polyvinylpyridine-N-oxide) which can coat the quartz surface.<sup>42,43,77</sup> Recent studies by the group of Dr Schins have confirmed the specific function of PVPNO in inhibiting the activation of the inflammasome and attenuating the grade of inflammation and lymphocyte influx.<sup>45</sup> Several hypothesis have been made on the binding mechanism.<sup>9,153</sup> The polar groups on the polymer exposing an oxygen atom with a dense negative charge are ideal points for strong H-bonding with those silanols identified as responsible for the inflammatory potential of silica. Functionalization of the silica particles, obtained by silanol reaction, is a very common procedure in nanomedicine, generally adopted to graft specific molecules at the surface. Organosilanes covalently bound to silanols have also been recently proposed as a good alternative to PVPNO.<sup>154</sup>

*Are quartz particles nongenerated by crystal fracturing pathogenic?* This is still an open question because accurate in vivo tests and large epidemiology studies are required to assess the absence of pathogenicity. We may however speculate on the various circumstances in which exposure to respirable crystalline silica non-obtained by fracturing might occur. The intact

synthetic quartz crystals described above are a very useful laboratory tool, but exposure to this type of silica is not likely to occur in the real world. Crystalline silica particles are formed when small silicon/silica clusters are exposed at extremely high temperatures such in the cases of flying ashes, volcanic ashes, heated biogenic silica sources, typically diatoms. In flying and volcanic ashes crystalline silica is usually concealed by mixed amorphous matter and toxicity, if any, has been ascribed to other components of the mixed dust.<sup>155</sup> The case of biogenic silica is different. Indeed, the product of diatoms transformation, diatomite earths or kieselgur, are very fibrogenic materials recognized as such long time ago<sup>70</sup> and also reported as carcinogenic in one large epidemiological study.<sup>156</sup> Most recent activities, such as burning sugarcane crops,<sup>157</sup> also appear to produce toxic particulate material. Diatoms undergo complex procedures aimed to eliminate the organic components and to prepare an appropriate material during which heating and grinding is required.<sup>158,159</sup> Thus we may infer that such procedures are likely to induce disorganized and reactive surfaces.

*Where we are with the toxicity of amorphous silica?* The toxicity of amorphous silica is much under debate, particularly in view of the application of SiNPs in nanomedicine. As far as our model is concerned the variability of outcomes in cellular and in vivo tests reflects variability in the distribution of surface functionalities in the same way as discussed for crystalline silica. The balance between acute inflammation and low biopersistence will establish specimen by specimen its ultimate toxicity. In vivo, amorphous silica mostly produce transient effects.<sup>3,46</sup> However, the presence in few cases of granulomatous lesions and silicotic nodules<sup>110,111,160,161</sup> suggest a precautionary approach in cases of exposures to such materials.

## 5. State of the art and future perspectives

The variability of quartz hazard can be fully justified by the large variations occurring in surface silanol distribution, which implies different levels of inflammation. However, besides inflammasome activation, the inflammatory response to silica is the result of several activated biochemical cascades such as the transcriptional regulators nuclear factor- $\kappa$ B (NF- $\kappa$ B) and activator protein-1 (AP-1), the activity of a number of soluble and cell-derived mediators, and oxidant-mediated triggering mechanisms,<sup>162-164</sup> which will also contribute to the variability in so far as related to some physico-chemical features of the particles. As instance, particle- and cell-derived ROS establish a robust oxidant milieu, meanwhile the major antioxidant defenses present in the bronchoalveolar space, ascorbate and glutathione, both react with the quartz surface – with silanols<sup>86</sup> and iron ions,<sup>85</sup> respectively. Such reactions deprive the alveoli of their defense against the released oxidants, exacerbating the oxidant stress and adding other surface functionalities to the list of those implied in toxicity.

Deconstructing the paradigm of crystallinity, however, does not mean that amorphous and crystalline silica could be similarly pathogenic. Amorphous silicas are *per se* much more soluble than crystalline silicas, moreover they are usually in ultrafine size (SiNP) which also facilitates dissolution. Therefore, only crystalline silica would be biopersistent and consequently establish in the alveoli the continuous cycle of AM ingestion, release of phagolysosomal content, recruitment and re-ingestion (as described in Figure 2) which is at the base of persistent inflammation. Moreover, crystalline forms only give rise to stable surface radicals and free radical-generating centers which may directly stress both macrophage and epithelial cells,<sup>58,82,95,147,162</sup> thus contributing to a parallel step yielding the pathogenic response.

As a whole, different particle characteristics contribute to the overall pathogenicity of a given source of silica; on the basis of this perspective, however, one could hypothesize that blocking one single step of the mechanism of toxicity induced by a specific and measurable particle feature, we could reduce or eliminate the pathogenic potential of a given silica particle. Particularly in the absence of surface functionalities, namely silanols that origin inflammation, the particle could be considered non-pathogenic if it will be confirmed that stopping the inflammatory step also the pathogenicity will stop. This is not an easy task, considering the complex reactivity of silica with water vapor, obviously ubiquitous in the real world. However, we have now the techniques – FT-IR and NMR spectroscopy – to identify the active silanol sites, which may become useful tools to predict the toxicity potential of a given silica dust, as well as to evaluate the efficiency of the various detoxification routes proposed. We suggest that this could be the way to carry out research aiming to a safe use of silica.

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### **Author Contributions**

The manuscript was written through contributions of both authors. All authors have given approval to the final version of the manuscript. The authors contributed equally.

### **Notes**

The authors declare no competing financial interest.

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## ABBREVIATIONS

AM, alveolar macrophage; AO, adverse outcome; AOP, adverse outcome pathway; AP-1, activator protein-1; IARC, International Agency for Research on Cancer; KE, key event; MIE, molecular initiating event; NF- $\kappa$ B, nuclear factor- $\kappa$ B; PVPNO, polyvinylpyridine-N-oxide; RBC, red blood cell; RNS, reactive nitrogen species; ROS, reactive oxygen species; RT, room temperature; SEM, scanning electron microscopy; SiNPs, silica nanoparticles; TEM, transmission electron microscopy.

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## Biographies



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