On the variability of silica toxicity: role of surface reactivity in membranolysis and in the activation of inflammatory pathways

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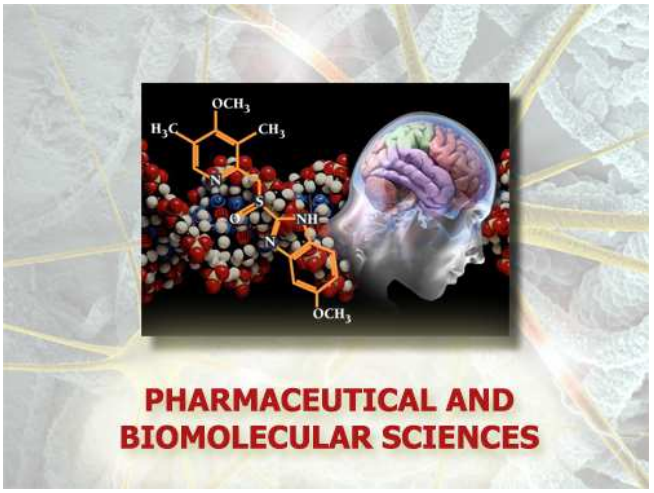

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Alla mia famiglia,
a zio Carlo

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List of Publications

Pavan C, Tomatis M, Ghiazza M, Rabolli V, Bolis V, Lison D, Fubini B: **In Search of the Chemical Basis of the Hemolytic Potential of Silicas.** *Chemical Research in Toxicology* 2013, **26:**1188-1198.

Pavan C, Rabolli V, Tomatis M, Fubini B, Lison D: **Why does the hemolytic activity of silica predict its pro-inflammatory activity?** *Particle and Fibre Toxicology* 2014, **11:**76

Pastero L, Turci F, Leinardi R, Pavan C, Monopoli M: **Synthesis of α-Quartz with Controlled Properties for the Investigation of the Molecular Determinants in Silica Toxicology.** *Crystal Growth & Design* 2016, JUST ACCEPTED

Turci F*, Pavan C*, Leinardi R, Tomatis M, Pastero L, Garry D, Anguissola A, Lison D, Fubini B: **The pathogenicity of silica: crystallinity and surface disorder. Revisiting the paradigm with synthetic quartz crystals.** *Particle and Fibre Toxicology*, SUBMITTED
* *Joint first authors*

Pavan C*, Polimeni M*, Tomatis M, Corazzari I, Turci F, Ghigo D, Fubini B: **"Artificial stone dusts": a new cause of an ancient disease. Particles generated by abrasion exhibit a radical activity higher than quartz and induce epithelial-mesenchymal transition in human bronchial epithelial cells.** *Toxicological Sciences*, SUBMITTED
* *Joint first authors*

Pavan C, Tomatis M, Turci F, Ghiazza M, Lison D, Fubini B: Z **potential as a tool to evidence impurities and heterogeneity in silanol acidity at the surface of quartz.** *Journal of Colloid and Interface Science*, TO BE SUBMITTED

Abbreviations

A549	Human alveolar epithelial cell line
AM	Alveolar macrophages
AOP	Adverse outcome pathway
BET	Brunauer Emmet Teller
DCS	Differential centrifugal sedimentation
DLS	Dynamic light scattering
DMEM	Dulbecco's modified Eagle's medium
DMPO	5,5-Dimethyl-L-pyrroline-N-oxide
DPBS	Dulbecco's phosphate buffered saline
ELISA	Enzyme-linked immunosorbent assay
ELS	Electrophoretic light scattering
EMT	Epithelial-mesenchymal transition
EPR	Electron paramagnetic resonance
FBS	Foetal bovine serum
FPIA	Flow particle image analysis
FTIR	Fourier transform infrared spectroscopy
HCA	High content analysis
J774	Mouse monocyte macrophage cell line
KE	Key events
LDH	Lactate dehydrogenase
LPS	Lipopolysaccharide
MIE	Molecular initiating event

MW	Molecular weight
qRT-PCR	Quantitative real time polymerase chain reaction
RBC	Red blood cell
ROS	Reactive oxygen species
SD	Standard deviation
SEM	Standard error of the mean / Scanning electron microscopy
TEM	Transmission electron microscopy
TGA	Thermogravimetric analysis
XRF	X-ray fluorescence

Contents

CHAPTER 1

Introduction

1.1. Silica and silica-correlated pathologies

1.1.1. The solid: silica

Silica is the chemical term related to a variety of species formed by covalently bound silicon and oxygen atoms combined in the stoichiometric ratio of 1:2 (SiO_2). The silicon-oxygen tetrahedron [SiO_4] is the basic unit of all silica specimens in which each silicon atom is surrounded by four oxygen atoms and each oxygen atom is shared by two tetrahedra (Fig.1.1).

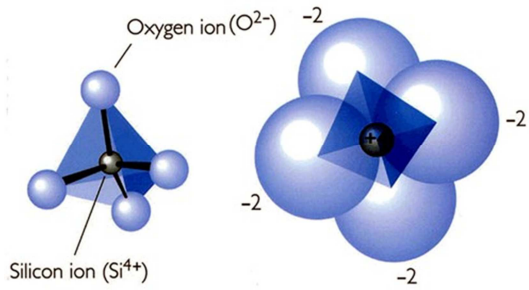


Fig.1.1 Silicon-oxygen tetrahedra—the building block of silica. Reproduced from [1].

While the [SiO_4] unit is rigid, the Si–O–Si angle that connects two tetrahedra can easily change as a function of temperature, pressure or geometrical constrains . Due to this high flexibility of the Si–O–Si angle a large number of silica-based materials are found in nature, from dense crystalline and amorphous structures (quartz or glasses) to porous systems (aerogels, sponges, zeolites and mesoporous material) [2].

Table 1.1 lists the most common forms of silica, classified as amorphous and crystalline, synthetic and natural. Crystalline silica is characterized by regular arrangements of the tetrahedra, and exists in a number of polymorphic forms depending on the different orientation and position of the tetrahedra. The main crystalline polymorphs of silica are quartz, tridymite, cristobalite, coesite and stishovite among which quartz is the most present in

nature (the 12% of the crust volume is represented by quartz) as it is the most thermodynamically stable at the earth surface. All the other polymorphs exist with metastability at room temperature and atmospheric pressure. Synthetic crystalline silica are not very common, but examples are some high silica forms (porosils) similar to the most common aluminosilicates called zeolites [3] or some hydrothermal cultured quartz crystals prepared *ad hoc* [4, 5].

Table 1.1 The most common forms of silica and their origin. Reproduced with modifications from [6]

Silica forms	***Origin***		
	Natural		**Synthetic**
	Mineral	Biogenic	
crystalline	α-quartz cristobalite tridymite		quartz crystals high silica zeolites
amorphous	vitreous silica hydrous silica (opal)	diatomaceous earth plants (sugar cane, rice, grain, wheat)	precipitated silica pyrogenic (fumed) silica silica gel silica glass

In amorphous structures the [SiO_4] units are random networks, the orientation of the bonds lacks any periodicity to form disordered systems. The amorphous natural forms can be of mineral origin, such as hydrous silica (e.g. opal) and vitreous silica, or of biogenic origin, that is silica originating in living matter. The two main biogenic silicas are diatomaceous earth, formed by the deposition in the earth of siliceous frustules of diatoms, and silica from crop plants such as sugar cane, rice and millet, which accumulate silica in their tissues to promote structural integrity and to protect against pathogens and insects.
Synthetic silica is generally produced in amorphous form, examples being pyrogenic, fumed and precipitated silica. In recent years, synthetic amorphous silicas have gained commercial importance in food and health care, especially as "drug delivery systems" [7] or as basis for the fabrication of engineered multifunctional systems [8], paving the way for a large body of studies assessing their biocompatibility and interactions with human body.

An extensive past or recent literature on the chemistry of silica has been published [2, 9-11], thus only the most striking features have been here reported, drawing from the aforementioned knowledge.

1.1.2. Silicosis, the most ancient occupational disease still in vogue

Silicosis is one of the most ancient occupational disease known to humankind [12]. It is a debilitating pulmonary disease which affects persons who chronically inhaled crystalline silica-containing dusts. The pathologic condition is marked by the development of fibrosis (pneumoconiosis) occurring in the lung parenchyma. The classical lesion is represented by the silicotic nodule, which is composed by a central hyaline necrotic area, laminar masses of collagen tissue and by inflammatory cells (macrophages, granulocytes, lymphocytes, polymorphonucleated cells) in periphery. These scar lesions are accompanied by a decrease in pulmonary functions: restrictive and obstructive pulmonary disease, respiratory failure, pulmonary hypertension that may result in systemic effects [13-15].

Since early days men were exposed to quartz dusts during mining, quarrying or working the surface layers of the earth, but only by the 18th - early 19th centuries the death from silicosis was recognized, and incidents have been reported over years associated to silica exposure [12, 16]. Hazardous exposure to silica dusts occurs in many industries and occupations, some listed in Table 1.2 [17], whenever workers cut, grind, crush, or drill silica-containing materials.

Table 1.2 Occupations and industries with silica exposures. Reproduced from [17].

Occupation	*Industry*
Sandblasting	Construction, painting, shipbuilding, ironworking
Miner	Mining underground
Miller	Silica flour mills
Ceramic worker	Pottery and ceramics
Glassmaker	Glass production
Granite quarry worker	Mining in quarries
Sand grinding	Industrial sand
Stone grinding	Granite industry
Casting, blasting	Foundry

Silicosis has thus represented one of the major occupational health issues until the 1990s when thanks to stricter regulations, improved working conditions, and the use of protective measures (e.g. dust control systems and respirators) there was a steady decline in death rate, especially in developed countries [13, 17, 18].

However, new outbreaks of silicosis still occur occasionally, and hazardous silica exposures have been newly documented during hydraulic fracturing of gas and oil wells [19], in sandblasting denim [20] and during fabrication and installation of engineered stone countertops [21, 22]. Moreover, in developing countries with inadequate regulation of exposed trades and poor surveillance, silicosis remains endemic [13, 23-25].

1.1.3. Other silica-related pathologies

Several epidemiological studies reported that inhalation of dusts containing crystalline silica is associated to lung cancer [17, 25]. Although the evidence of a direct carcinogenic effect of silica has long been debated [26], quartz and cristobalite were classified in 1997 by the International Agency for Research on Cancer (IARC) as Group 1 carcinogens, as there was sufficient evidence for carcinogenicity in experimental animals and humans [27].
Moreover, silica exposure has been associated to some autoimmune diseases such as systemic lupus erythematosus, rheumatoid arthritis, systemic sclerosis and chronic renal disease [6, 28-31].

1.2. The two paradigms of silica toxicity: crystallinity and the variability of silica hazard

1.2.1. Crystallinity as determinant feature of silica toxicity

Silicosis as well as the other silica-related pathologies have traditionally been associated to exposure to crystalline silica dusts, thus suggesting crystallinity as the prerequisite feature of hazardous silica particles. Even IARC classified as carcinogenic for humans only “inhaled crystalline silica in the form of *quartz* or *cristobalite* from occupational sources" [27]. Since early epidemiological and experimental studies there was evidence that crystalline silica may cause inflammation, fibrosis and lung cancer [32-37].
An aspect highly underestimate by toxicological experts is that native uncontaminated quartz surfaces, made up of perfect crystal faces, very rarely are in direct contact with living matter, if not expressly synthesized for laboratory investigations [5, 38-40]. Usually, workers are exposed to crystalline silica dusts of respirable size generated either by grinding macroscopic crystals of quartz of mineral origin or by heating/calcining biogenic silicas (e.g. diatomaceous earth) [41]. Mechanical comminution of quartz crystals yields a different distribution of crystallographic surfaces forming irregular conchoidal fractures [42-44] and a perturbed external amorphous layer (known as Beilby layer) [41, 45-48]. Quartz, contrary to many brittle solids such as asbestos, does not possess crystallographic planes of weakness (cleavage planes). Thus breakage causes curving fracture surfaces which are also typical of amorphous material (e.g. silica glass), but in the case of quartz, the conchoidal fracture is characterized by a

concatenation of numerous microscopic patches of single crystallographic surfaces. Images showing conchoidal fractures on a big crystal of quartz or on a microscopic quartz particle obtained by ball milling of larger crystals are reported in Fig. 1.2. Moreover, TEM images of a very pure quartz dust prepared by milling (Fig. 1.3) show that the crystal lattice is only evident in the bulk of the quartz crystal, while a non-ordered thick and displaced fractures are appreciated at the edges.

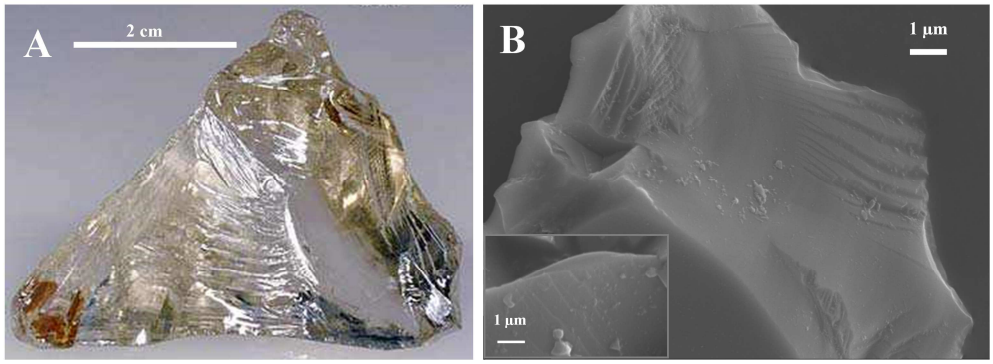


Fig.1.2 Images of a big crystal of quartz (A) and a micrometric quartz particle laboratory prepared by ball milling of larger quartz crystals (B) both exhibiting conchoidal fractures.

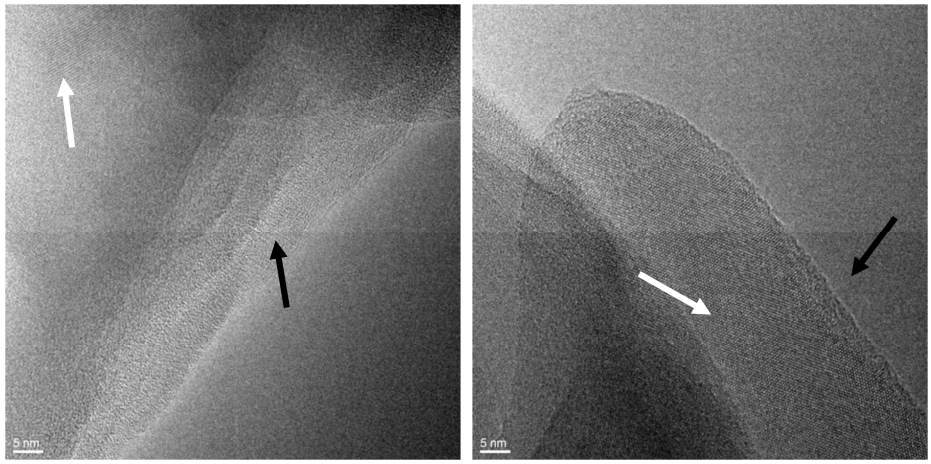


Fig.1.3 TEM images of a pure quartz dust obtained by milling. Crystallinity (white arrows) is clear only in crystal bulk, while an amorphous layer and conchoidal fractures are evident at the edges (black arrows).

While the toxicity of crystalline silica is well documented, amorphous silica has always been considered less or non-toxic. At the time of 1997 IARC classification, some studies reported some cytotoxic effects on cells [49, 50], but *in vivo* the inflammatory response was transient [51, 52], with no

progressive lung fibrosis [53-55]. For these reasons in 1997 the final statement by IARC on amorphous silica was of inadequate evidence of carcinogenic effects in humans and in experimental animals [27]. However, amorphous silicas were studied less than crystalline silicas [27, 55, 56], and after this judgment, the trend has been inverted due to the large increase in the use of amorphous nanosilica for medical, cosmetic and food applications. Although the toxicity induced by amorphous silica is still controversial, it is now clear that also amorphous silicas are not equally toxic, similarly to what happens with crystalline ones [57]. In particular a critical distinction in terms of toxic effects is emerging between amorphous silicas obtained by wet process (precipitated, colloidal, mesoporous silica, silica gel) or by pyrolysis (pyrogenic or fumed silica). Pyrolytic silica, unlike the precipitated ones, often resulted highly reactive towards cell systems [58-61] and in experimental animals [56, 59, 62, 63].

1.2.2. The variable toxicity of silica

Crystallinity has always been reported as the main determinant of silica toxicity. However, the different silica polymorphs showed marked variations in their toxicity, either in *in vitro* endpoints such as DNA damage and transforming effect [64], membranolysis [44] and cytotoxicity [65], and in *in vivo* clearance, inflammation and fibrosis [36, 66-68]. Indeed, the uncommon polymorphs coesite and stishovite were found inert compared to quartz, cristobalite, and tridymite [69]. Moreover, not all the sources of the same crystalline polymorph (e.g. quartz) were found equally pathogenic [25, 27, 70] and these differences were reflected in their biological reactivity reported in experimental studies [71-73]. As an example, in a complex inter-laboratory study on the toxicity of a large panel of commercial quartz samples deriving from different sites of the European quartz industry, quartz dusts showed a highly variable ability to induce cytotoxic, genotoxic, inflammatory and fibrogenic effects in both *in vitro* and *in vivo* tests [74-77]. This large variability of the toxic effects of silica was also exceptionally recognized by IARC, who specified that, for crystalline silica, "carcinogenicity in humans was not detected in all industrial circumstances studied" [27]. This is also the main reason of the prolonged debate on the identification of silica toxicity/carcinogenicity which is still underway. The differences found in carcinogenicity to humans and in experimental studies among different quartz sources led the scientific community to recognize a "variability of quartz hazard" [70]. Later, the same notion on the variability of silica toxicity was proposed again for nanosilicas – all fully amorphous – by Napierska and co-workers in a review titled "The nanosilica hazard: another variable entity" [57].

A silica particle, whether crystalline or amorphous, micron or nano-sized, acts as particulate toxicant thus, unlike molecular toxicants, its toxicity is not predictable from the chemical composition alone [71]. Even if all types of silica are characterized by the same chemical composition (SiO_2), each silica sample exhibits peculiar physico-chemical features and surface functionalities which are function of the origin, the mechanical and the thermal history of the dust, and which may evolve with time. Thus, regardless of the crystal structure, each property may impart a different toxic potential to a silica particle [41, 71, 78].

1.3. Physico-chemical properties of silica determining its toxicological variability

In the light of the biological evidence that not all types of silica are toxic when inhaled, studies focused on the physico-chemical bases of the variability of silica pathogenicity. Pioneering studies in the 1950s already indicated a possible role of chemistry (e.g. crystallinity, aluminium ions) underlying the variable toxicity of silica [36, 79, 80]. Then, the research continued in search of one single property that would have explained the toxic effects of silica. Among the properties investigated, the features of crystalline silica not present on the amorphous species appeared the most reliable. In Fig. 1.4 the physico-chemical features of silica suspected to contribute to its toxic potential are highlighted. A more comprehensive insight of each property in relation to silica toxicity follows up.

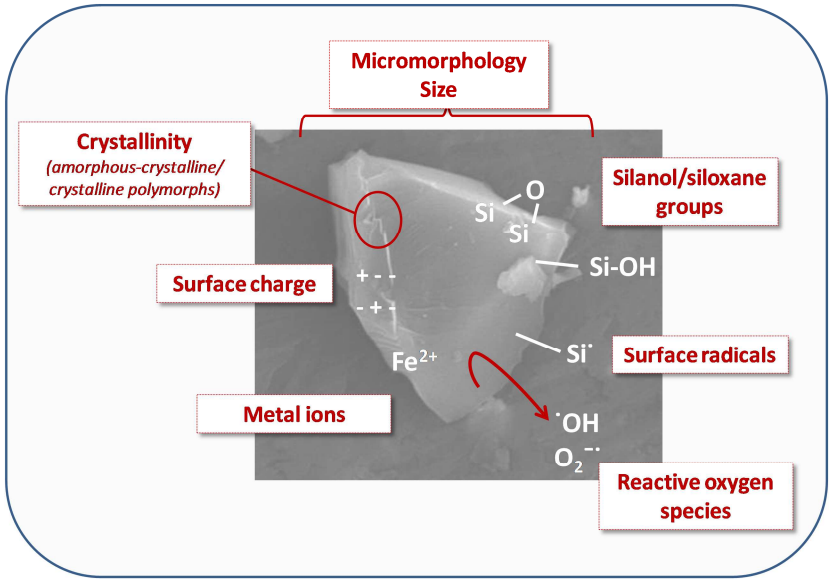


Fig.1.4 Physico-chemical properties of silica determining the large variability of silica toxicity.

1.3.1. Crystallinity and crystalline polymorphs

The different crystal habit of crystalline silica and the variability on the (regular/disordered) arrangement of the tetrahedra [SiO_4] composing the bulk give rise to different crystalline polymorphs and amorphous specimens, and therefore it is the primary cause of the toxicological variability of silica, as thoroughly explained in the previous paragraph.

1.3.2. Micromorphology and size of the particles

Inhalation of silica particles is restricted to "respirable particles", with a mean aerodynamic diameter < 5 μm [81].

The micromorphology of silica particulates is dependent upon the way they are formed. Pathogenic crystalline silicas are usually obtained by grinding processes and for this reason they exhibit irregular surfaces, with acute spikes and sharp edges. Their particle size is usually heterogeneous, ranging from few microns to nano-scale silica fragments sticking on the surface of the bigger ones [71] as showed in Fig. 1.5A. Amorphous silica (considered less pathogenic), such as those obtained by wet precipitation (e.g. synthesized following Stöber procedure) or pyrogenic silica generated at high temperatures, show smooth and roundish particles of almost the same size [57] as shown in Fig. 1.5B. The size may be tuned depending on the synthetic conditions to obtain fine or nano-particles [82].

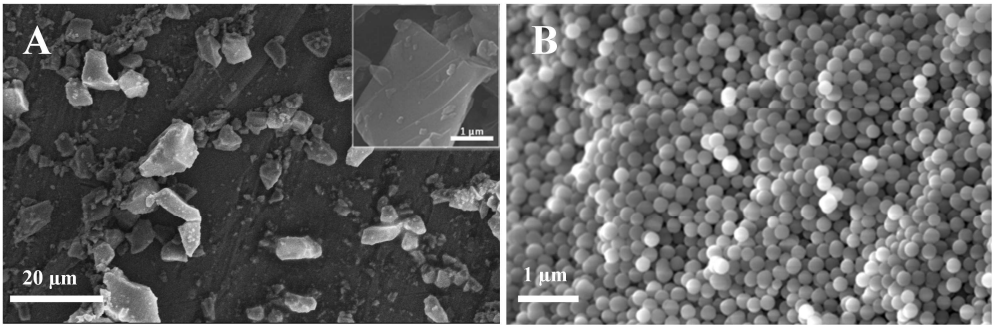


Fig.1.5 Micromorphology and size of a commercial quartz (Min-U-Sil 5) obtained by milling (A) and of an amorphous silica (Stöber silica) obtained by wet synthesis (B).

Particle size has also been claimed as relevant feature in membranolysis and lung inflammation even if with contrasting findings [5, 83, 84].

1.3.3. Reactive oxygen species and surface radicals associated to freshly fractured surfaces

During fracturing of macroscopic crystals to obtain crystalline silica dusts, silicon-oxygen bonds are cleaved via homolytic or heterolytic routes generating highly reactive surface sites: dangling bonds ($Si^{\bullet}$ and $SiO^{\cdot}$,

reactive surface radicals with an unpaired electron in a p orbital) and surface charges (Si^+ and SiO^-) [78, 85]. Once Si–O bonds are broken, a complex surface reconstruction takes place and dangling bonds tend either to recombine to form strained reactive Si–O rings [2], or they react with atmospheric components yielding reactive oxygen species (ROS) such as hydroxyl radicals, superoxide anion and peroxides [85-87]. ROS are highly reactive species which have been associated with some steps of the pathogenic activity of silica [70, 71, 78, 87-90]. Strained three-membered $(SiO)_3$ rings (3MRs) have also been proposed as determinants of the peculiar toxicity of amorphous silica produced by pyrolysis [59].

Freshly fractured surfaces are more reactive than aged ones in both chemical terms and in the biological response elicited by silica [41, 87, 89, 91-94]. Moreover, the state of the surface depends markedly on the grinding procedure and the components of the environment in which the grinding took place [95, 96]. A dry oxygen atmosphere favours formation of surface radicals and ROS, while a wet one assists full surface hydration at broken bonds, with no yield in surface reactive forms. Moreover, grinding can insert contaminants derived from the components of the jar, modifying silica reactivity [97]. Metal contaminants, in particular iron, can catalyse the formation of ROS, especially hydroxyl radicals via a Fenton mediated-reaction. Therefore, also free radicals produced as a consequence of the reaction with some components of the aqueous medium in which particles are suspended may be added to the oxidative stress generated by surface radicals. In solution, both surface radicals and metal ions may constitute centres able to generate free radicals [98]. At the solid-liquid interface many mechanisms may occur generating free radicals [99]. Two common free radical-generating reactions are of interest [97, 99]:

(1) a "Fenton-like" reaction in presence of hydrogen peroxide generating hydroxyl radicals:

$$M^{n+} + H_2O_2 \longrightarrow M^{(n+1)+} + HO^{\bullet} + HO^{-}$$

Where M is usually iron present either as a mineral component or as a trace contaminant, but may be replaced by any electron donor.

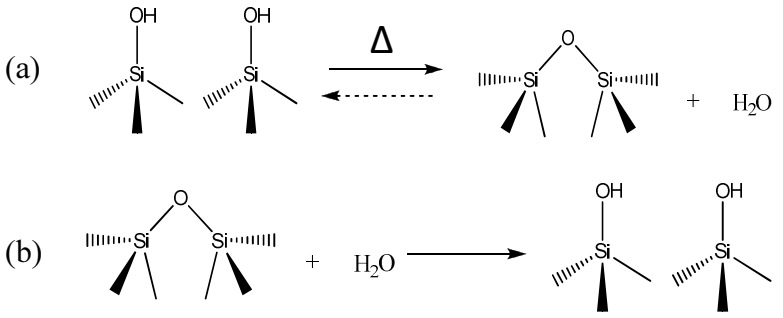
(2) homolytic cleavage of hydrogen-carbon bonds generating carbon centered radicals:

$$X + H\text{-}R \longrightarrow (HX) + R^{\bullet}$$

Reaction (1) and (2) are independent and originate from different surface properties of the particles.

1.3.4. Degree of hydrophilicity associated to surface silanol and siloxane functionalities

The surface of silica is mostly made up of hydrophilic and hydrophobic patches. Silanols (−SiOH) impart hydrophilicity whereas siloxanes (−Si−O−Si−) hydrophobicity [100]. There are great differences in terms of hydrophilicity among the various crystalline polymorphs and in general crystalline silica is more hydrophilic than amorphous one [100]. The extent of silanols can be modified upon thermal treatment. Upon heating, silanols condense into siloxanes with elimination of water, as shown in Scheme 1, part (a).



Scheme 1 Condensation of silanols into siloxanes upon heating (a) or surface hydration by siloxane opening (b).

This reaction progressively converts hydrophilic surfaces to hydrophobic ones [78]. When cooling down under ambient conditions, some water uptake takes place, with partial reconversion of siloxanes into silanols. However, high temperature and prolonged heating stabilize surface siloxanes with inhibition of rehydroxylation. The opposite treatment is the hydrothermal treatment in which silica surface reacts with water vapour at temperatures lower than 250° C in condition of high humidity (Scheme 1, part (b)).This treatment is performed to increase the external layer of silanols [100]. In addition to some specific treatments (e.g. thermal treatments), surface hydration and hydroxylation can take place in a moist atmosphere with kinetics dependent on several factors including crystallinity, the synthetic process and the thermal history of the silica sample [78, 100]. Thus, silanols and siloxanes functionalities are continuously in evolution with slow or fast kinetics.

The degree of hydrophobicity/hydrophilicity of the surface is an important determinant of toxicity. It is thought to regulate coating by endogenous material [101-103], membranolysis [104, 105], cytotoxicity and modifies translocation in various biological compartments [106, 107].

1.3.5. Surface charge

Surface charges may be originated in air by mechanical grinding following heterolytic rupture of silicon-oxygen bonds [41], or by deprotonation of surface silanols when the silica particle is suspended in an aqueous solution [108]. In a simplified approach, silanols at the silica surface may be treated as week monoprotic Brønsted acid, and the pH of the surrounding media directly determines the charge of the silica surface following the equilibrium:

$$-SiO^- + H_2O \leftrightarrows -SiOH + OH^-$$

As a consequence, in an alkaline environment the vast majority of silanols will be dissociated with a net surface charge markedly negative (silanolate, ≡ Si−O$^-$). As the pH decreases, the protonated form is favoured and the overall surface charge becomes less negative. At pH < 2 all silanols are in the protonated form and the net charge of quartz is virtually zero.

The surface charge of silica has been largely overlooked in the field of particle toxicology, and often this is not mentioned among the physico-chemical properties affecting silica toxicity. Only few studies reported an essential role of the negative surface charge of silica in the interaction with cell membranes [109, 110]. With the advent of nanomaterials, the surface charge gained more attention as crucial determinant of the interactions with endogenous proteins [111, 112]. Adsorbed proteins can form a "corona" around the nanoparticles [113-116] thus altering their recognition, cellular uptake and cytotoxicity [117-120]. Highly charged surfaces adsorb more proteins, while zwitterionic or neutral organic coating prevent adsorption of serum proteins [112].

1.3.6. Contaminants and coating

External factors such as contaminants, coating molecules and chemical modifications can alter the reactivity of the silica surface [71].

Metal impurities (aluminium, iron, copper, sodium, potassium, calcium and titanium) are widely present in silica dusts, especially in the mineral ones [121]. Aluminium and iron are the most present because of their charge/radius ratio and the geometrical features of the surface oxygen groups [122]. They are also able to substitute for silicon in the tetrahedral framework. Commercial quartz derived from silica sand, sandstone and quartzite are affected by a high degree of impurities, usually about 5% but it may be up to 25% [121]. The reactivity of silica is extremely modified in presence of contaminants. For example aluminium-doped quartz showed a reduced membranolysis [123] and *in vivo* inflammation [124]. Coating with aluminium lactate strongly decreased the adverse biological effects of quartz [125-128]. On the contrary iron ions, through a Fenton reaction, are able to

increase ROS production and for this reason they enhance DNA damage, cell transformation and pulmonary reactions [129]. However, it has been demonstrated that iron deposited on surface of quartz reduced cytotoxic and inflammatory responses in alveolar macrophages [130]. One of the most common and relevant contaminant associated to quartz is coal. When in intimate contact whit it, such as in coal mine dusts or in ground mixtures, quartz loses its pathogenic potential [70]. Even experimental studies showed that carbon associated to quartz may reduce its biological reactivity [131].
Various external coating agents such as lipid surfactants, proteins, organosilane, and the polymer polyvinyl-pyridine-*n*-oxide (PVPNO) were used to reduce silica toxicity [70]. In particular, the polymer PVPNO was a strong inhibitor of silica membranolysis [122], cytotoxicity and fibrogenicity [132, 133] as it is a strong hydrogen-bonding agent, able to bind silanol groups at the quartz surface [41].

1.4. The cellular mechanisms underlying silica-induced lung injury

In spite of massive work since the early 1950s on the pathogenicity of silica-related diseases, the specific mechanisms that induces silica-induced inflammation and fibrosis at the origin of silicosis and possibly lung cancer, as well-as the role of each physico-chemical property in triggering a specific biological event are still partially unclear. At the beginning of the 2000s, the discovery of the protein complex "inflammasome" [134] which triggers the inflammatory reaction in response to a number of endogenous and exogenous stimuli, including particulate toxicants such as uric acid crystals, asbestos, and silica [135], imparted a depth improvement in the understanding of the mechanism of inflammation of silica. Several mechanisms of the pathogenicity of silica have been proposed during subsequent years [69, 70, 136], which are all summarized in the one proposed by IARC for the carcinogenicity of crystalline silica [25] (Fig. 1.6), that are not here reported for brevity. Only some general and key aspects of the biological activity of silica, relevant for the purpose of this thesis are reported below.

1.4.1. General aspects of the biological actions of silica within the lungs

Respirable silica particles (aerodynamic diameter < 5 μm), if not cleared by the mucociliary escalator, attain the alveolar space where they may be coated by phospholipids and surfactants. The presence of the particles as foreign bodies elicits a response of the immune system primarily by the alveolar macrophages (AM) which have been postulated to trigger the chain of events leading to chronic lung fibrosis and cancer, via an inflammatory-mediated mechanism [137, 138] (Fig.1.6).

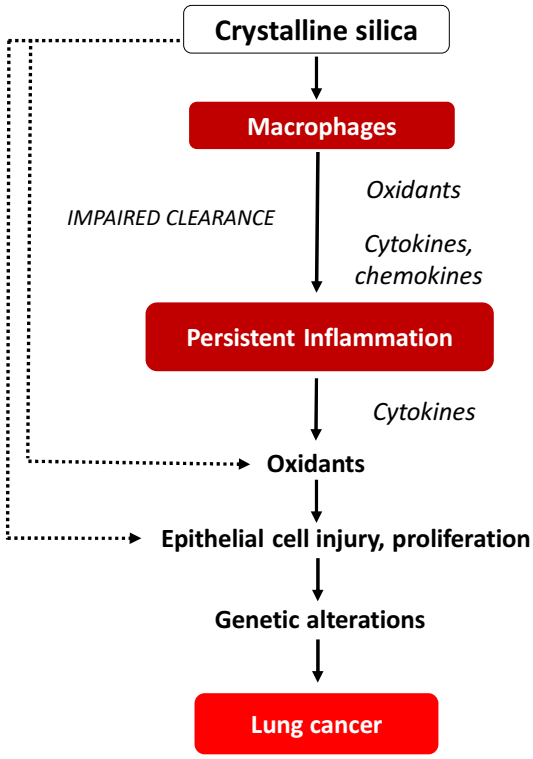


Fig.1.6 Proposed mechanisms for the carcinogenicity of crystalline silica in rats. Reproduced with modifications from [25].

AM express a variety of cell-surface receptors that can bind silica (the most relevant the type A scavenger receptor SR-A, including SR-A(I/II) and MARCO) modulating silica uptake and possibly leading to apoptosis or macrophage activation [136, 137]. Once having been identified, the surface coated particle is engulfed by AM into a phagosome that fuses with a lysosome to form a phagolysosome. Phagolysosomes contain a number of enzymes that serve to degrade ingested material into low molecular weight products; in the case of a particulate, enzymes digest organic molecules adsorbed on the surface, thus cleaning the particle surface [117, 137]. Depending upon the physico-chemical characteristics of the silica particle, macrophages succeed in particle clearance or, on the contrary, macrophage activation occurs. Particles contained within the phagolysosome can interact with the inner membrane and cause phagolysosome destabilization and lysis [139]. Lysosomal enzymes and undigested particles are then released into the cytoplasm enhancing macrophage death or inflammatory pathways [140-142]. Activation of macrophages triggers the transcription and release of a

large variety of factors such as cytokines, growth factors, alarmins, reactive oxygen (ROS) and nitrogen (RNS) species, arachidonic acid derivatives, each of whom will/may play a part in the pathogenic process [143]. Mesenchymal cell-stimulating cytokines will lead to the increase in mesenchymal cells and products in the interstitium that are characteristic of silicosis [144-146].

Subsequent ingestion-reingestion cycles accompanied by a continuous recruitment of AM and accumulation of other immune cells such as neutrophils contribute to the sustained inflammation elicited by silica. Target cells such as bronchial and alveolar epithelial cells will then be affected by both alveolar macrophages products or by the particle itself resulting in activation, mutation and/or cell death [27, 138]. This is accompanied by increased interstitial translocation of silica and accumulation in the lymph nodes [136] with concomitant reduction of clearance [68, 147] and accumulation of a dose in the lung. Factors are released which stimulate fibroblasts and increase collagen synthesis [136]. Repeated macrophage death, fibroblast stimulation and abnormal collagen synthesis promote the silicosis process [81, 122].

1.4.2. Bases of the interaction of silica with membranes

In the above mechanism, understanding the interaction of a silica particle with the cell membrane is a key factor because this is the first interaction of the particle with the AM, and membrane damage is one of the starting step triggering adverse effects of silica. The cell membranes more involved in the interaction with silica particles are the outer plasma membrane and the inner phagolysosome membrane. While the interaction with the outer plasma membrane is likely mediated by the protein corona adsorbed on the surface of silica [148], when the particle is engulfed by AM and internalized into phagolysosomes, the surface coating is removed from the particle surface by lysosomal enzymes [117]. The unmasked silica surface can then react with the inner surface of the phagolysosomal membrane, possibly causing membrane damage. Several theories have been proposed during years to explain the molecular interactions between silica and membrane surfaces, which have been previously reviewed in [41] (p. 442-444) and are thus only listed here:

- the hydrogen-bonding theory [81, 149], based on the hydrogen-donor ability of the silanol groups [41], that can interact with hydrogen acceptors such as phosphate ester groups of phospholipids or proteins of biomembranes;
- the electrostatic interaction theory, for which the negative surface charge of the ionized silanol groups ($-SiO^-$) can bind positively charged groups such as quaternary alkylammonium groups of membrane phospholipids [122];

- the oxidative stress theory, based on the recognized ability of quartz to generate surface reactive species, free radicals and cause lipid peroxidation [150, 151].

1.4.3. Recent insights into the inflammatory pathways induced by silica

Inflammation is the result of the activity of a number of soluble and cell-derived mediators and of activated biochemical cascades in response to cellular irritants.

The inflammatory reaction caused by silica particles is characterized by activation of multiple cell types including AM, lymphocytes and (bronchial and alveolar) epithelial cells in producing numerous inflammatory mediators such as reactive oxygen species (ROS), reactive nitrogen species (RNS), alarmins (IL-1α, HMBG-1, S100, heat shock proteins) inflammatory cytokines (such as TNF-α, IL-1β, IL-8, IL-6, IFNγ,) chemokines (e.g. MIP-2), growth factors (e.g. TGF-β) and arachidonic acid derivatives (prostaglandin-E_2) [136, 143, 152].

Beside two of the main transcriptional regulators of pro-inflammatory responses to silica that are nuclear factor-*k*B (NF-*k*B) and activator protein-1 (AP-1) [152, 153], recent evidence demonstrated that NLRP3 inflammasome is pivotal in the development of lung inflammation induced by silica [140, 154]. Inflammasome assembly leads to the activation of the inflammatory caspase-1 that controls maturation and release of the interleukin IL-1 family [135], in particular IL-1β one of the most involved pro-inflammatory cytokines in silica related disease [155]. The IL-1 β stimulates alveolar cells, including epithelial and endothelial cells and fibroblasts to release cytokines (e.g.IL-1, TNF-α), TGF-β, PGE_2, nitric oxide, platelet activating factors, and it also positively auto-regulates its own synthesis via NF*k*B binding activity [156].

The upstream mechanism of particle-mediated NLRP3 inflammasome activation include potassium efflux and ROS production [157]. A different hypothesis is based on phagolysosomal destabilization by ingested particles and that enzymatic content of lysosomes released into the cytosol activate NLRP3 inflammasome. Inhibition of the lysosomal protease cathepsin B has been proven to reduce IL-1β secretion [140]. Release of active IL-1β into the extracellular environment requires also a transcriptional step to induce the production of the biological inactive pro-IL-1β which is then processed by NALP3 inflammasome. The transcriptional signal and NALP3 inflammasome may be mediated by transcriptionally active pattern recognition receptors that can detect pathogen components (PAMPs, e.g. lipopolysaccharide, LPS) [158] or by NF-*k*B activating stimuli such as TNF-α [159]. It has been recently demonstrate that early release of alarmins (such

as IL-1α, and to a lesser extent IL-33) by silica-stimulated macrophages can induce pro-IL-1β production and subsequent inflammatory reaction [160].

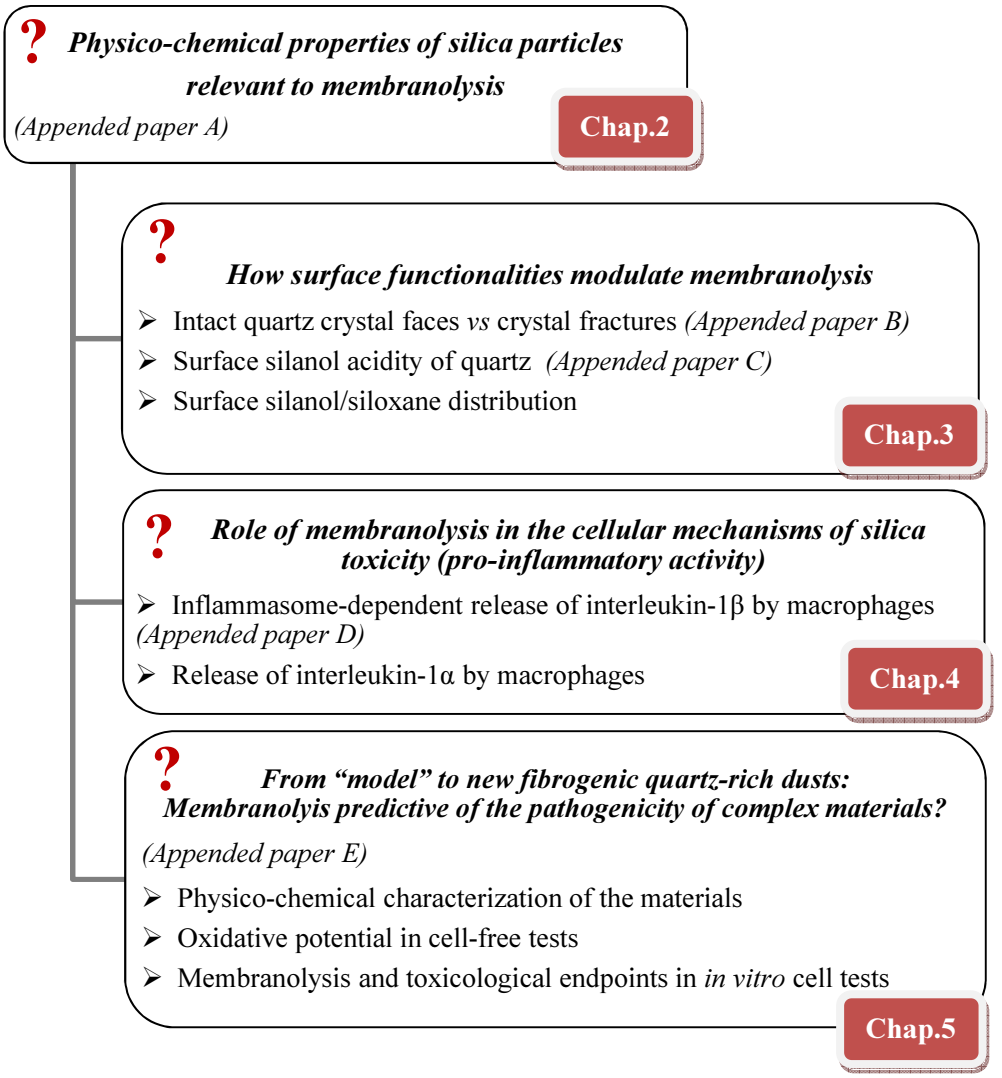
1.5. Objectives and outline of the work

To the current state of the art it is evident that more than one physico-chemical property contributes to the pathogenic potential of silica and that several physico-chemical features are involved in each step of the cascade of cellular/biomolecular events at the base of the mechanism of silica. For instance, several inflammatory pathways and mediators (e.g. cytokines, alarmins, chemokines) may be activated by silica, each of whom may be influenced by different silica features. In spite of a large body of studies, the role of each physico-chemical property in triggering a specific biological response, as well as the molecular mechanisms leading to the inflammatory and fibrotic condition at the origin of silicosis are still partially unclear.
The complexity of the cellular mechanisms leading to silica pathogenicity and the high heterogeneity of the silica surface make the real system highly intricate, thus models to simplify this complexity are needed. On the one hand models of solids with tuneable and easily measurable properties, on the other hand models of particle-cell interactions would be useful to unravel this complexity and to investigate the physico-chemical properties and molecular mechanisms of silica toxicity.
The *fil rouge* of this work was the use of both:

- silica models, such as tailored synthetic quartz crystals or commercial quartz and amorphous silicas of controlled surface properties, which can be modulated by specific physico-chemical treatments, up to more complex silica-containing particulates;
- a model of cell membranes, i.e. red blood cells (RBCs). RBCs play no part in the pathogenesis of silicosis, but they have traditionally been employed as simple and convenient model to investigate the membranolytic effects of toxicant particulates, as one of the starting step triggering detrimental effects of silica.

As summarized in Scheme 2, the objectives of this thesis were:
1) to identify the physico-chemical features of silica particles inducing membranolysis (as a possible toxicological target of silica toxicity) by using a large set of model and commercial silica particles. 2) To better clarify how the properties identified in step one can modulate the interaction with membranes; in this regard, we focused on the role of intact as-grown crystal faces of quartz opposed to fractured surfaces, on the role of the silanol acidity of quartz (which may be affected by metal contaminants), and of the surface distribution of silanols and siloxanes. 3) To correlate the membranolytic potential with some of the cellular mechanisms involved in the inflammatory response triggered by silica particles, in particular we examined the inflammasome-dependent release of the interleukin-1β and the release of the alarmin interleukin-1α by macrophages. Finally, 4) to

investigate the physico-chemical properties, oxidative potential in cell-free tests and *in vitro* toxicity of new quartz-rich dusts found extremely pathogenic in humans, the so-called "artificial stone". Among the toxicological endpoints investigated *in vitro*, we concentrated on membranolysis, cytotoxicity and occurrence of the epithelial-mesenchymal transition in bronchial epithelial cells.



Scheme 2 Flow chart of the experimental work plane.

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CHAPTER 2

Physico-chemical properties of silica particles relevant to membranolysis

2.1. Summary

Context: the lysis of red blood cells (hemolytic activity) was one of the first test used to evaluate the membranolytic potential of inhaled particles and fibres in a toxicological perspective. Even if the hemolytic activity of silica particles has long been investigated and sometimes associated to several markers of pathogenicity, the molecular mechanisms and the reasons why hemolysis differs according to the silica type were still unclear.
Experimental design: we set out a new approach by combining in a single research work a large number of crystalline and amorphous silica samples differing in size, origin, morphology and surface chemical composition. The hemolytic activity of all silica samples was assessed and compared relying on a physicochemical property of interest.
Main achievements contributing to the knowledge on silica toxicity: the present study pointed out the relevance of silica surface functionalities i.e. silanols and siloxanes in the interaction with membranes, regardless of the form (crystalline or amorphous) or the size of the silica particle. In particular, hemolysis by silica particles appear not merely related to silanol density but rather to a specific arrangement of silanols, silanolates and siloxane groups at intermediate surface hydration. Moreover, some other physico-chemical properties, essential in the pathogenic response to silica, such as crystallinity and ability to generate free radicals resulted not involved in silica hemolytic activity.
Through this study we broadened the knowledge on the physicochemical determinants of silica membranolytic potential which may be useful both in understanding at a molecular level the mechanisms of interaction silica-cell membranes and in assisting the rational design of biocompatible materials.

2.2. *Appended paper A (published in Chemical Research in Toxicology):*

"In search of the chemical basis of the hemolytic potential of silicas"

Author's contributions

CP performed part of the physicochemical treatments and characterization of the silica samples at the Dept. of Chemistry, Centro Scansetti, University of Turin under the supervision of MT, MG and BF. She carried out the hemolysis experiments at the Université catholique de Louvain (Brussels) under the supervision of VR and DL. CP analysed the data and wrote the manuscript with the contribution of all the other authors.

CHAPTER 3

Modulation of the membranolytic effect by silica surface functionalities: a deeper investigation

3.1. Summary

Context: based on the results obtained from the first study, the silica surface functionalities i.e. silanols and siloxanes found relevant to membranolysis were here examined from three different points of view consisting in i) fractures of crystalline surfaces, ii) distribution of acidic surface sites, and iii) various silanol patterns, in order to clarify the specific surface arrangement that induces membranolysis.
Experimental design: the hemolytic activity was assessed in a study i) comparing synthetic as-grown quartz crystals exposing intact surfaces *vs* fractured ones in a series of cellular and acellular assays relevant for the pathogenicity of silica particles; ii) investigating the ζ potential *vs* pH of a set of quartz particles with different levels of purity; iii) in which the silanol pattern of a model silica was selectively tuned and controlled via specific thermal treatments.
Main achievements contributing to the knowledge on silica toxicity:
(i) As-grown quartz crystals did not show biological reactivity (e.g. membranolysis) in a series of toxicologically relevant tests contrary to fractured quartz. In synthetic as-grown quartz crystals, a regular distribution of the silanols at the particle surface was shown, and fracturing caused a loss of the long-range order, likely creating disordered surface functionalities and reactive silanol patches. A new paradigm not based on crystallinity was proposed for the pathogenic activity of silica particles.
(ii) Two different evolutions of ζ plot (ζ potential *vs* pH) were observed on a large set of quartz dusts and were associated to pure and contaminated surfaces. The degree of heterogeneity in acidity of silanols on the pure surfaces may be evaluated from the steepness of the ζ plot and well-correlated with membranolysis of quartz particles, as by reducing the surface heterogeneity (via chemical treatments) the hemolytic activity decreased.

(iii) Via specific thermal treatments, the surface silanol pattern of a model silica was tuned and controlled by FTIR spectroscopy. The maximum of membranolysis was attained when strongly H-bonded silanols were suppressed leaving only weakly interacting and isolated silanols. These results suggest that a surface characterized by a greater disorder, involving an extensive quota of weakly interacting silanols is better able to interact with membranes, as silica surfaces containing silanols mutually engaged in long-range interactions (such as H-bond chains) or only isolated silanols does not efficiently interact with membranes.

The results of these three studies are consistent with the notion that membranolysis and more in general the biological reactivity, hence toxicity of a silica particle, are related also to *surface silanol disorganization*. For the first time in the study of silica toxicity this notion has been proposed as one of the main determinants of silica pathogenicity.

3.2. *Appended paper B (submitted to Particle and Fibre Toxicology):*

"The pathogenicity of silica: crystallinity and surface disorder. Revisiting the paradigm with synthetic quartz crystals"

Author's contributions

Most of the research was performed by CP and RL at the Dept. of Chemistry, Centro Scansetti, University of Turin under the supervision of FT and BF and with the expert collaboration of LP of the Dept. of Earth Sciences, University of Turin. CP carried out the HCA and CPS experiments at the University College of Dublin (Ireland) under the supervision of SA and DG, and performed the hemolysis experiments at the Université catholique de Louvain (Brussels) under the supervision of DL. CP wrote the manuscript in collaboration with FT, BF and DL.

3.3. *Appended paper C (to be submitted to Journal of Colloid and Interface Science):*

"Z potential as a tool to evidence impurities and heterogeneity in silanol acidity at the surface of quartz"

Author's contributions

CP carried out the ζ potential experiments at the Dept. of Chemistry, Centro Scansetti, University of Turin under the supervision of MT and BF and performed the hemolysis tests at the Université catholique de Louvain (Brussels) under the supervision of DL. CP processed the data and wrote the manuscript in collaboration with FT, BF and DL.

3.4. Investigation on the nature of the silanol/siloxane distribution imparting a membranolytic activity to a silica particle

3.4.1. Introduction

In the first part of this thesis, the surface distribution of silanols and siloxanes was found crucial for the membranolytic activity induced by silica particles and measured as the damage to red blood cell (RBC) membrane [1]. A relevant result of that study evidenced for a large set of crystalline and amorphous silicas, that silica-induced membranolysis was not merely relatable to silanol density, but rather appeared modulated by a - not yet clarified - specific distribution of silanols (−Si-OH) (whether undissociated or dissociated) and siloxanes (−Si−O−Si−). Moreover, such silanol distribution was suggested to lead the activation of some pro-inflammatory pathways involving phagolysosomal membrane destabilization in alveolar macrophages in response to silica particle exposure [2].

The surface properties of silica are mostly established by the nature, density and distribution of silanols and siloxanes, which impart to the particle the hydrophilic and hydrophobic character, respectively [3, 4]. In general, hydroxylated silica surfaces are characterized by several types of silanol families (isolated, geminal, vicinal) (Fig. 3.1a) and different patterns of hydrogen (H)- bonded silanols (terminal or interacting through H-bonds) (Fig. 3.1b) [5].

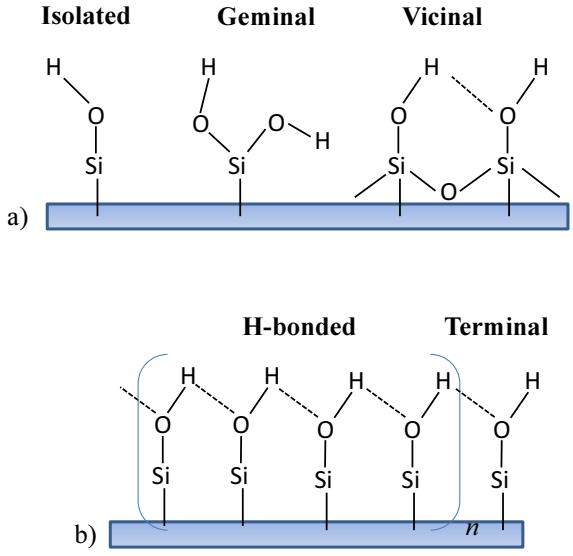

Fig. 3.2 Silanol groups (a) and patterns (b) at silica surface.

The presence of H-bonds depends on the density and the positions in space of the surface silanol groups [5], which in turn are the results of the underlying solid structure, thus the polymorph, the synthetic procedure, and all the external factors affecting the silica surface. When silanols are not involved in mutual H-bond, they are free to establish H-bond interactions with H-bond donor or acceptor sites largely present on biomolecules and membranes (e.g. secondary amide groups or nitrogen/oxygen in an amide of membrane proteins and phosphate groups of phospholipids) [6-8] or via electrostatic interactions when dissociated (with positive terminals such as quaternary alkylammonium ions of phosphatidylcholines or sphingomyelins) [8].
To identify the surface silanol/siloxane distribution which gives the most efficient interaction in causing membranolysis is not an easy task both for technical difficulties (such as create self-supporting pellets suitable for FTIR investigations, the best technique to probe silanol surface distribution) and for the great variability of the physico-chemical features among different silica sources. Thus, we here selected a model sample of silica whose silanol distribution was relatively easy to measure, tune and control by thermal treatments, taking advantage from previous studies conducted by the group of Prof. Martra [9]. Characterization of the surface silanol profile was carried out by Fourier Transform Infrared (FTIR) spectroscopy and membranolysis was assessed by RBC lysis (hemolysis).

3.4.2. Materials and methods

Silica sample
The silica sample selected was the Aerosil OX 50 (A50), a non-porous pyrogenic silica from Degussa (Frankfurt A.M., Germany) with primary particle size of 40 nm and specific surface area of 50 m^2/g.

Thermal Treatments
A50 was heated in air at 450° (A50/450°C) and 700° C (A50/700°C) for 2.30 h in order to modulate silanol distribution [3].

Infrared Spectroscopy
The technique refers to [10]. The samples used were compressed to give self-supporting pellets suitable for infrared measurement and placed in a quartz cell equipped with CaF_2 windows. Spectra were recorded with a FTIR spectrometer (Bruker IFS28, equipped with a DTGS detector, resolution of 4 cm^{-1}), by accumulating 150 co-added scans to obtain a good signal-to-noise ratio. The cell used for experiments was attached to a conventional vacuum line (residual pressure $\leq 5\times10^{-5}$ mbar) allowing adsorption-desorption experiments to be carried out *in situ.* The spectra were reported in absorbance after scattering correction and were normalized with respect to

the intensity of signals at 1980 and 1870 cm^{-1} due to combination of silica bulk framework modes. This was made in order to render differences in intensity, independent of different thickness among pellets. Spectra were recorded at beam temperature (.ca 50° C) after outgassing to remove water molecules adsorbed on the surface. Experiments with D_2O were applied to the outgassed samples after several freeze-pump-thaw cycles. D_2O adsorption and desorption cycles were repeated until no changes occurred in the spectra.

Hemolysis assay on human erythrocytes

Erythrocytes were purified from fresh human blood obtained by healthy volunteer donors not receiving any pharmacological treatment. The method refers to [11]. Briefly, erythrocytes were purified from blood (collected in vacutainer tubes containing K_2EDTA as anticoagulant) by centrifugation at 1200 x g for 10 min (Heraeus, Megafuge 11R, Thermo Scientific, USA), they were washed four times with 0.9% NaCl (Eurospital S.p.a., Trieste, Italy), and finally suspended in 0.9% NaCl at the final concentration of 5% by volume. Silica samples were dispersed at the concentration of 200 cm^2/ml in 0.9% NaCl and sonicated during 2 min (40 W, Sonoplus HD 2070, Bandelin, Berlin, Germany). Serial dilutions of the starting dispersion were performed to the final concentrations used for experiments (200, 100, 50, 25, 12.5 and 6.25 cm^2/ml). Particle suspensions were distributed in quadruplicate in a 96-well plate (150 µl/well) and the RBC suspension was then added (75 µl/well). Negative and positive controls consisted in 0.9% NaCl and 0.1% Triton-X 100, respectively. The plate was incubated at room temperature on an orbital plate shaker for 30 min and then centrifuged at 1200 rpm for 5 min (Heraeus, Megafuge 1.0R, Thermo Scientific, USA). Supernatants were finally transferred to a new plate (75 µl/well) and the absorbance of the haemoglobin released was determined at a wavelength of 540 nm on a microplate reader (Benchmark Plus, Bio-Rad, Hercules, USA).

Statistical analysis

Data are presented as mean ± standard deviation (SD). Differences between groups were analysed by one-way analysis of variance (ANOVA) with Tuckey's post hoc test. Differences with p value < 0.05 were considered statistically significant.

3.4.3. Results

The effect of thermal treatments on the surface silanol distribution of a pyrogenic silica

The characterization of the surface silanol distribution was carried out by FTIR spectroscopy before and after exchange with deuterated water (D_2O) on the pristine sample (A50) and the same sample heated at 450°C (A50/450°C) and at 700°C (A50/700°C) as reported in Fig. 3.2A.

The FTIR spectrum of the pristine A50 (outgassed at r.t.) in the hydroxyl (OH) spectral region (ca. 3800-3000 cm^{-1}) shows bands relative to modes of vibration of different OH families [10, 12], as better shown in Fig. 3.2B:

- a broad band at lower frequencies (3100 - 3600 cm^{-1}) assigned to silanols which mutually and strongly interact via H-bonds;
- one region at intermediate frequencies (ca. 3600 - 3742 cm^{-1}) associated to silanols weakly interacting among them (e.g. via van der Waals or weak H-bonding) such as terminal silanols of long-range H-bonded chains (3720 cm^{-1});
- a narrow absorption peak at ca. 3747 cm^{-1} assigned to isolated non-H-bonded silanols.

Upon heating at 450° C, the broad band assigned to strongly interacting silanols (3100 - 3600 cm^{-1}) was clearly reduced with respect to the pristine A50; conversely, the bands relative to weakly interacting silanols (3600 - 3742 cm^{-1}) and isolated ones (3747 cm^{-1}) were preserved. H-bonded silanols were partially converted into isolated ones as the peak at 3747 cm^{-1} increased. The differences in silanol population between the pristine and 450° C-heated A50 were better evidenced by the D_2O exchange (dotted spectra of Fig. 3.2A). This procedure allows to probe only the surface OH groups available to interact with the D_2O. This causes a shift towards low wavenumbers [13, 14]. Moreover, by D_2O exchange intraglobular hydroxyls (i.e. inter-particle OH groups, generated by the aggregation of secondary particles during pyrolytic synthesis) can be revealed. They are inaccessible to D_2O, thus continue to oscillate at high frequencies and are attributable to the band near 3650 cm^{-1} [13].

By heating at 700° C, even the band assigned to weakly interacting silanols disappeared in favour of the band due to isolated silanols. Thus both the strongly (H-bonded) and the weakly (van der Waals) interacting silanols were removed by heating at 700° C. While at 450° C only the H-bond interacting silanols were selectively suppressed.

The treatment performed in these specific experimental conditions allowed to obtain irreversible surface modifications (i) after a few days of exposure to air and (ii) after contact with water vapour pressure and subsequent outgassing (data not here shown).

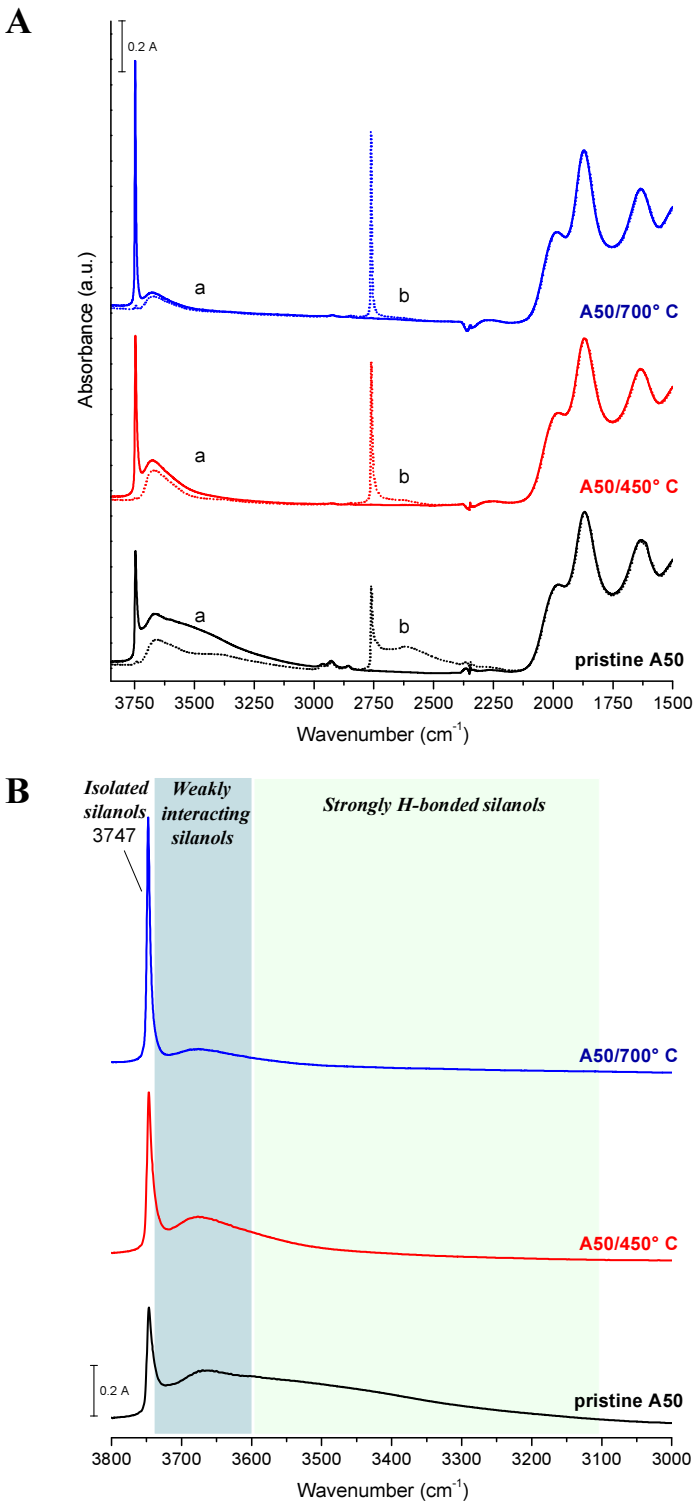

Fig. 3.2 (See legend on next page)

Fig. 3.2 FTIR profiles of the three outgassed A50 materials: pristine A50 (bottom, black), heated at 450° C (middle, red) and at 700° C (top, blue). (A) Solid line: spectra outgassed after contact with water vapour pressure (a), dotted line: spectra outgassed after contact with D_2O vapour pressure (b). (B) Magnified view of the OH spectral region of the silica samples outgassed after contact with water vapor pressure and relative OH stretching modes assigned to the various silanol groups.

The effect of thermal treatments on the membranolytic ability of a pyrogenic silica

The hemolytic activity *vs* increasing concentrations of the pristine and the heated A50 samples is reported in Fig. 3.3. A dose-dependent increase of the hemolytic activity was observed for all samples. Upon heating at 450° C, the hemolytic activity of A50 significantly increased with respect to the pristine A50. On the contrary, a significant decrease of the hemolytic activity of A50 was observed for the sample heated at 700° C.

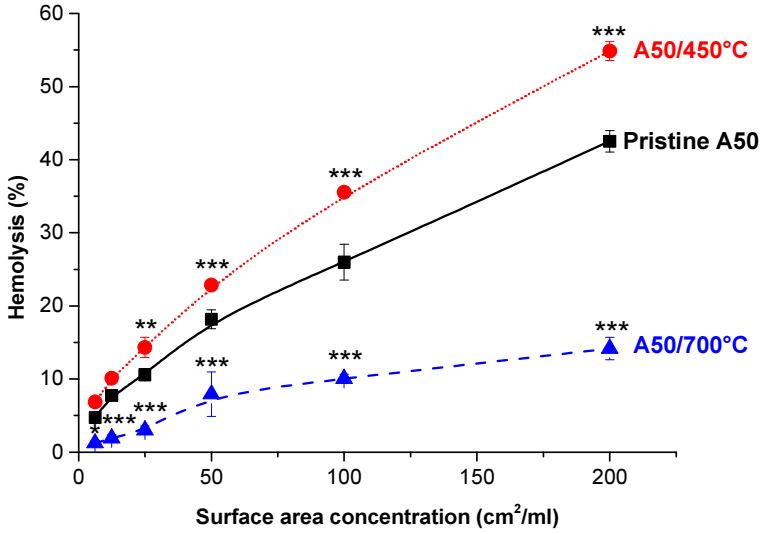

Fig. 3.3 Hemolysis (%) induced by pristine A50 (black), A50 heated at 450° C (red) and at 700° C (blue). Purified human red blood cells (RBC) were incubated with increasing concentrations of silica samples. Values are mean ± SD from one single experiment performed in quadruplicate. Data from one representative experiment out of three giving similar results is depicted. *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$ pristine *vs* heated.

3.4.4. Discussion

The pyrogenic silica A50 was selected as model of silica particle to clarify how hemolysis can be modulated by specific surface silanol/siloxane patterns in virtue of the possibility to control its surface features. Moreover, the toxicity of this amorphous silica has been recently recognized *in vitro* in more than one study, as opposed to amorphous silica obtained by wet synthesis [15-18]. Pyrogenic silica is usually less hydrophilic than amorphous silica obtained by wet precipitation [1, 10, 13] because of the high temperature attained during the synthetic process, causing a decrease of the number of OH groups by condensation [5].

To tune surface silanol distribution, two heat treatments at different temperatures have been performed. When silica was heated at 450° C the majority of H-bonded silanols was condensed into siloxane species and only weakly (via van der Waals forces) bonded or isolated silanols were left. Further heating at 700° C allowed to skim again silanol populations, and only isolated silanols were selectively obtained. As a result, three different surface situations were achieved, from the fully hydroxylated A50, where the whole set of silanol families were present, to a surface containing only isolated species. As the hemolytic activity of A50 increased after the treatment at 450° C, while strongly decreased by heating at 700° C, weakly interacting silanols appear as the silanol population most involved in silica-induced hemolysis.

Some computational studies have shown that the surface energy of silica is mostly determined by the properties of the H-bonds formed at the silica surface. In general, the strongest the H-bond interactions are, the lower the surface reaction energy is [19]. We could therefore deduce that formation of H-bonds between mutual interacting silanols, forming long-range interactions like H-bond chains, and acceptor sites of biomolecules is energetically unfavourable. This was further supported by a later study of Musso et al. [20] who showed that the energy of interaction of silica with H_2O and NH_3 molecules anti-correlated with the density of surface silanols. This was explained considering that pre-existing H-bonds on the silica surface needed to be broken to establish new H-bonds with the adsorbate. The interaction strength of the silica surface-biomolecule thus depends on the presence of available H-bonding sites only if not mutually H-bonded.

Similarly, a heated silica surface containing only isolated silanols, does not efficiently interact with biomolecules. A surface characterized by a greater disorder, involving an extensive portion of weakly interacting silanols thus seems to be better able to interact with membranes.

However, we may not exclude that modification of the particle surface by heating will result in different aggregation of the primary particles, hence

affecting the hemolytic activity of A50 as for other amorphous nanosilicas [21]. Hence, future perspectives of the present study will aim to better clarify if/how aggregation would influence the hemolytic activity of this sample. Moreover, giving the great interest of this study, we might expand the research to some other silica models (e.g. a crystalline silica).

3.4.5. References

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CHAPTER 4

Investigating the role of membranolysis in the inflammatory responses induced by silica

4.1. Summary

Context: this section investigates how hemolysis caused by silica particles may be related to newly emerging mechanisms of pro-inflammatory responses induced by silica particles. Among the triggering factors of this inflammatory response, the release of the interleukins IL-1β and IL-1α is essential.
Experimental design: a panel of selected silica particles with different surface and bulk physico-chemical characteristics and hemolytic activity was used to evaluate *in vitro* the release of interleukins IL-1β and IL-1α by macrophages. An extra effort was carried out in order to clarify the mechanisms underlying IL-1β and IL-1α release by macrophages exposed to different silica particles.
Main achievements contributing to the knowledge on silica toxicity: after ascertaining the involvement of the inflammasome and the requirement of phagocytosis and lysosomal rupture for IL-1β activation, the results showed for the silica particles under study a significant correlation between IL-1β release and their hemolytic activity. On the contrary, no correlation was found between hemolytic activity and IL-1α release. Based on the knowledge that IL-1β release requires labilization of the phagolysosomal membrane after particle phagocytosis and that IL-1α release results from damage of the outer plasma membrane, RBC membrane lysis appears associated to phagolysosomal destabilization rather than plasma membrane injury. However, from the present study, the mechanism of IL-1α release appears more complex and finely regulated by additional steps related to gene transcription and protein production that are also required before IL-1α release. Thus, the present research helped to clarify why hemolysis is often predictive of the inflammatory activity of (silica) particles, and it contributed to shed light on the activation mechanisms of such essential inflammatory mediators of silica toxicity.

4.2. *Appended paper D (published in Particle and Fibre Toxicology):*

"Why does the hemolytic activity of silica predict its pro-inflammatory activity?"

Author's contributions

CP contributed to the experimental design and carried out the hemolysis and *in vitro* experiments at the Université catholique de Louvain under the supervision of VR and DL. CP collected and characterized the samples at the Dept. of Chemistry, Centro Scansetti, University of Turin with the support of MT and BF. CP analysed the experimental results and drafted the manuscript with the essential inputs of all other authors.

4.3. Release of the alarmin IL-1α by silica-exposed macrophages: from the mechanisms of IL-1α activation to the physico-chemical properties of silica

4.3.1. Introduction

The interleukin-1-alpha (IL-1α) is a pro-inflammatory cytokine of the IL-1 family. Unlike most cytokines which are up-regulated upon stimulation, IL-1α is constitutively present in resting cells under homeostatic conditions [1, 2] and is part of the alarmin group [3-5]. Alarmins are a class of heterogenic endogenous mediators which initiate, amplify and sustain the inflammatory response [6]. They are passively released by necrotic cells (but not by apoptotic cells) or actively secreted by stimulated leukocytes. IL-1α, together with some other constitutive cytokines such as IL-33 [7] and High Mobility Group Box 1 (HMGB1) [8, 9], belongs to the class of "dual-function cytokines" [1, 10] as, next to some extracellular functions promoting leukocyte recruitment and activation [11, 12], it also plays important intracellular tasks at the nuclear level [13, 14].

IL-1α is synthesized as a pro-form (31 kDa) like IL-1β, but contrary to the latter the precursor is already active [15]. The Ca^{2+}- activated protease calpain, associated to the plasmatic cell membrane or in the cytosol, can cleave the pro-form into the mature form (17 kDa) [16-18]. The necessity of IL-1α cleavage is not clear since both the precursor and the mature forms can bind the IL-1R1 receptor (also bound by IL-1β) present on plasma membrane of resident alveolar macrophages thus promoting the genetic transcription of inflammatory mediators via the NFkB pathway [19]. However, it has been shown that the affinity of the mature IL-1α for its receptor is increased [1]. Pro-IL-1α may be cytosolic, membrane-bound, nuclear or released into the extracellular environment. The membrane-bound IL-1α is anchored into the plasma membrane of active monocytes operating juxtacrine signal transduction [1, 20, 21]. IL-1α may act as a transcriptional factor. Following activation of Toll-like receptors (TLR), it translocates into the nucleus and induces the expression of a large portfolio of pro-inflammatory genes (e.g. IL-6, IL-8 and pro-IL-1β) [14]. During apoptosis, pro-IL-1α is concentrated into the nucleus in dense foci preventing its release, while during necrosis or pyroptosis it is passively released into the extracellular medium [2, 22]. The calpain-mediate cleavage is unusual, thus the precursor is the most abundant form in many cell types (e.g. endothelial, epithelial cells, fibroblasts, immune cells) [10]. The mature IL-1α form is only found in the extracellular medium. After cleavage, the N-terminal pro-piece containing the NLS (Nuclear Localisation Sequence) is removed, and IL-1α loses the ability to translocate into the nucleus but retains the capacity to bind the IL-1R1 receptor.

IL-1α and IL-1β appear to have the same biological activity as they both bind the same receptor; however, they are expressed at different steps of the inflammatory response and, as a consequence, contribute to recruit different myeloid cells. It has been demonstrated that IL-1α starts and mediates the early stages of sterile inflammation inducing neutrophil infiltration, while IL-1β amplifies the later inflammatory response promoting the recruitment and retention of macrophages [5, 11]. Knowledge on IL-1α is more limited compared to IL-1β, and some of the pathways and mechanisms of IL-1α release have yet to be understood [1, 23]. Unlike IL-1β, it is not clear to what extent the pro-IL-1α or the mature IL-1α are released into the extracellular medium and if an active mechanism of secretion is involved for the mature form as suggested only for myeloid cells. The activation of the inflammasome has also been thought to be involved in IL-1α secretion, although this process is poorly characterized and also dependent on calpain [24]. As reported [25], contrary to pro-IL-1α, secretion of mature IL-1α requires additional activation of the inflammasome and Caspase- 1.
In a previous experimental research [26], IL-1α was found as one of the leader cytokines in acute lung inflammation induced by silica particles. In particular, it has been shown that following instillation of mice with silica, lung stocks of IL-1α mostly contained in alveolar macrophages, were rapidly released (1h) in the alveolar space and that the release of IL-1α preceded and triggered the expression and secretion of IL-1β. Moreover, the *in vitro* capacity of a set of silica particles to release IL-1α from macrophages was well correlated with their *in vivo* inflammatory activity [26].
Based on the findings that: 1) macrophages represent an appropriate cell model to use as they are the main IL-1α-secreting lung cells, and *in vitro* especially murine J774 macrophages possess high constitutive levels of IL-1α (compared to fibroblast MLg or murine alveolar epithelia cell LA-4) [26]; 2) the release of IL-1α follows plasma membrane rupture [1], the aims of this work were: 1) to elucidate the *in vitro* mechanisms of IL-1α release by macrophages exposed to silica particles, and 2) to correlate the release of IL-1α with hemolysis, as in both cases membrane damage is claimed to be implicated. The murine macrophage J774 cell line was used as *in vitro* model to investigate the mechanisms of IL-1α release. The commercial quartz Min-U-Sil 5 (Qz-1), with known inflammatory and fibrogenic potentials [27] was selected as a reference silica particles. A broad set of silica particles with different physico-chemical features was also considered to evaluate the correlation between IL-1α release and RBC membrane damage. At the beginning of the project we also tuned particle concentrations, cell numbers/well, the use or not of foetal bovine serum (FBS), to determine the paramount exposure conditions (data not reported here).

4.3.2. Materials and methods

Silica samples

The silica samples used, whose main characteristics are reported in Table 4.1, were: (Qz-1) the commercial microcrystalline α-quartz Min-U-Sil 5, largely used in studies of experimental silicosis and lung cancer [27], purchased from U.S. Silica Co. (Berkeley Springs, WV, lot number 15062696); (Qz-2) the Min-U-Sil 5 quartz heated in vacuum at 800°C for 2 h to reduce surface hydrophilicity [28]; (Qz-P) obtained by grinding a very pure quartz crystal from Madagascar in a planetary ball mill (Retsch S100, GmbH, Haan, Germany) for 3 h (70 rpm), then in the mixer mill (Retsch MM200) for 1 h (27 Hz). The grinding process was performed in an agate jar to keep silica free from impurities; (n-Qz-syn) synthetic highly pure quartz crystals in submicron size obtained through hydrothermal synthesis using HNO_3 as polymerizing agent [29, 30]; (MSS) an amorphous silica (Ångström spheres) made up of monodispersed silica spheres purchased from Fiber Optic Center Inc. (New Bedford, MA); (A50) aerosil OX 50, a nonporous fumed silica from Degussa (Frankfurt A.M., Germany); and (VS) a vitreous silica obtained by grinding a very pure silica glass (Suprasil) produced for optical applications in a ball mill (agate jar) for 3 hours (70 rpm).

Chemical reagents

Dulbecco's modified Eagle medium (DMEM) GlutaMAX, fetal bovine serum (FBS), penicillin-streptomycin (10,000 U and 10,000 mg/ml) and Dulbecco's phosphate buffered saline (DPBS) were obtained from Invitrogen (Merelbeke, Belgium). The cell proliferation reagent WST-1 was purchased from Roche Applied Science (Vilvoorde, Belgium) and the Triton X-100 from Flucka (Buchs, Switzerland).

Cell culture and particle exposure

The J774 murine macrophage cell line (ATCC#TIB-67) was grown to pre-confluence in cell culture flasks in DMEM GlutaMAX supplemented with 10% FBS, penicillin (100 U/ml) and streptomycin (100 μg/ml). Before particle exposure, J774 macrophages were seeded in 96-well plates (50.000 cells/well) in DMEM GlutaMAX supplemented with penicillin (100 U/ml) and streptomycin (100 μg/ml) and allowed to adhere for 4h at 37°C in a 5% CO_2 atmosphere. Particles were heated at 200°C for 2 h just prior to suspension in order to sterilize them and to inactivate any trace of endotoxin. Silica suspensions were prepared just before use in serum free DMEM GlutaMAX and sonicated in a bath during 2 min. Silica suspensions or serum free DMEM GlutaMAX (control) were distributed in six to four replicates in cell culture plates to the final concentrations of 5, 10, 20 and 40 cm^2/ml and incubated for 1, 3, 6 or 18h.

Table 4.1 Main physicochemical properties and hemolytic activity of the silica samples

Sample	Origin	SSA[a] (m^2/g)	Morphology[b]	Size	Metal Impurities (% oxides)	Free radical generation[f]	%Hemolysis (at 100 cm^2/ml)[g]	Ref.
Min-U-Sil 5 (Qz-1)	ground natural mineral, commercial	5.2	acute spikes and edges, conchoidal fractures	1.7 ± 0.7 μm[c]	Al 1.4 Fe 0.06	$OH^{\cdot}$: ++ $COO^{\cdot -}$: ++ (almost stable kinetic)	+++	[31-35]
Min-U-Sil 5/ 800°C (Qz-2)	ground natural mineral	5.2	acute spikes and edges, conchoidal fractures	1.4± 0.6 μm[c]	nd	$OH^{\cdot}$: +++ $COO^{\cdot -}$: ++ (almost stable kinetic)	++	[31, 32]
Pure quartz (Qz-P)	ground natural mineral	3.8	acute spikes and edges, conchoidal fractures	1.4 ± 1.2 μm[c]	absent	$OH^{\cdot}$: + $COO^{\cdot -}$: + (increasing kinetic)	+++	[31, 34, 36]
As-grown quartz crystals (n-Qz-syn)	synthetic crystals	5.7	Intact and regular crystals	310 nm[d] PdI 0.236	absent	$OH^{\cdot}$: ++ $COO^{\cdot -}$: absent	+	[29, 30]
Monodispers -ed silica (MSS)	Stöber-like synthesis, commercial	4.4	Spherical smooth	1.1 ± 0.5 μm[c]	absent	absent	absent	[31, 33, 36]

Aerosil 50 (A50)	pyrogenic silica, commercial	50	Non porous spherical smooth primary particles, forming aggregates in suspension	40 nm[e] (primary particles) 317 ± 0.273µm[d]	absent	absent	+++	[31, 33]
Vitreous Silica (VS)	ground fused silica	3.1	Irregular morphology with acute spikes and edges	1.6 ± 1.2 µm[c]	absent	$OH^{\cdot}$: + $COO^{\cdot -}$: absent	+++	[31, 32, 36]

[a]Specific surface area (SSA) evaluated by BET (Brunauer, Emmet and Teller method).

[b]Evaluated by Scanning Electron Microscopy (SEM).

[c]Determined by FPIA (Flow Particle Image Analysis) which measures the average diameter expressed as circle equivalent (CE) diameter ± SD.

[d]Obtained by Dynamic Light Scattering in water.

[e]Obtained by TEM (Transmission Electron Microscopy).

[f]Measured by electron paramagnetic resonance (EPR) spectroscopy and using DMPO as trapping agent. Hydrogen peroxide or sodium formate were used as target molecules to generate respectively hydroxyl ($HO^{\cdot}$) or carboxyl ($COO^{\cdot -}$) radicals.

[g] +, 0-10%; ++, 10-30%; +++, 30-60%. Data reported refer to previous experiment reported in [30, 31] or were verified/determined following the protocol described in [31].

Assessment of cytotoxicity

At the end of the exposure period, the cytotoxic response was evaluated by measuring mitochondrial activity by the WST-1 assay (5% WST-1 reagent in DMEM) following the procedures indicated in [32] or by measuring cell membrane integrity by the lactate dehydrogenase (LDH) release assay [37]. LDH activity was measured spectrophotometrically into culture supernatant (LDH_{medium}) and in cell lysates (LDH_{cells}) after addition of 0.1% Triton X-100 (Saint-Luc University Hospital, Brussels, Belgium). Cell viability was calculated according to the formula:

% cell viability = $(LDH_{cells} / (LDH_{cells} + LDH_{medium}))*100$.

Determination of IL-1α

IL-1α released into culture supernatant or accumulated into cells was measured by enzyme-linked immunosorbent assay (ELISA). For intracellular IL-1α, cell pellets were lysed by adding 200 µl of 0.1% Triton X-100. An ELISA kit (DY400, R&D Systems, Wiesbaden-Nordenstadt, Germany) was used according to manufacturer's instruction, with a detection limit of 5 pg/ml.

RNA extraction and quantification

Total RNA was extracted using TriPure isolation reagent (Invitrogen) according to manufacturer's instructions. Extracted RNA was reverse transcribed with random hexamers and M-MLV reverse transcriptase (Invitrogen). Synthesized cDNA was amplified by quantitative real time polymerase chain reaction (qRT-PCR) using SYBR Green PCR Master Mix (Applied Biosystems, Life Technologies Corporation, CA, USA) on a StepOnePlus Real-Time PCR System (Applied Biosystems). Sequences of interest were amplified by using as forward primers (Invitrogen): CGG CTA CCA CAT CCA AGC AA (mouse 18S rRNA) and TTG AAG ACC TAA AGA ACT GTT ACA GTG AA (mouse IL-1α), while as reverse primers: ATA CGC TAT TGG AGC TGG ATT ACC (mouse 18S rRNA) and GCC ATA GCT TGC ATC ATA GAA GG (mouse IL-1α). Gene expression of the house-keeping gene 18S rRNA was used for normalization [26].

Statistical analysis

Data are presented as mean ± standard error of the mean (SEM). Differences between groups were analyzed by one-way analysis of variance (ANOVA) with post hoc Dunnett's test. Differences with p value < 0.05 were considered statistically significant.

4.3.3. Results

Exposure of J774 macrophages to quartz causes a rapid induction of IL-1α gene expression and accumulation

In order to better clarify the mechanisms of IL-1α production and release by J774 macrophage cell line exposed to quartz particles, we first measured IL-1α gene expression (Fig. 4.1A) and protein accumulation (Fig. 4.1B) in cells exposed to increasing concentrations of quartz (Qz-1) at different time points (1 to 18h). Already one hour after quartz exposure, IL-1α transcript and protein levels were significantly increased in a dose-dependent manner. These effects were more markedly evident after 3h of incubation. After 6h, the IL-1α protein content in quartz-exposed macrophages was still increased compared to the control, and the highest production was reached after 18h. However, at 18h the intracellular IL-1 α level was strongly reduced at the highest dose of quartz, suggesting that stored IL-1α was released into extracellular medium after membrane injury.

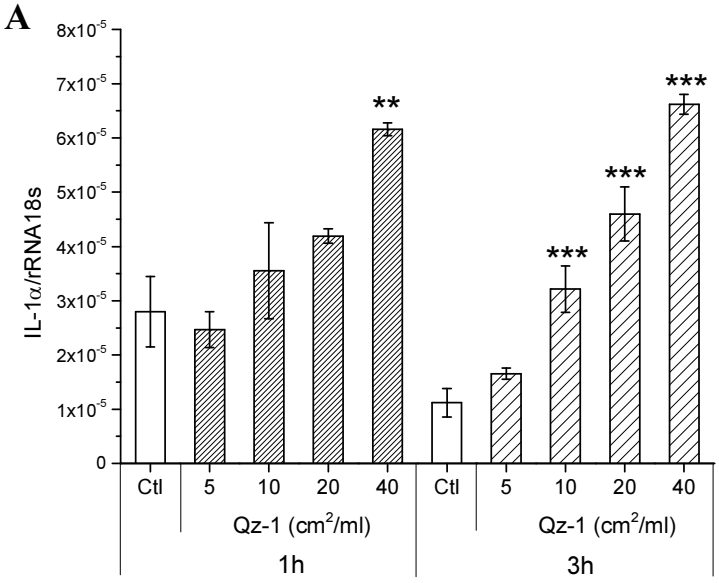

Fig. 4.1 (See legend on next page)

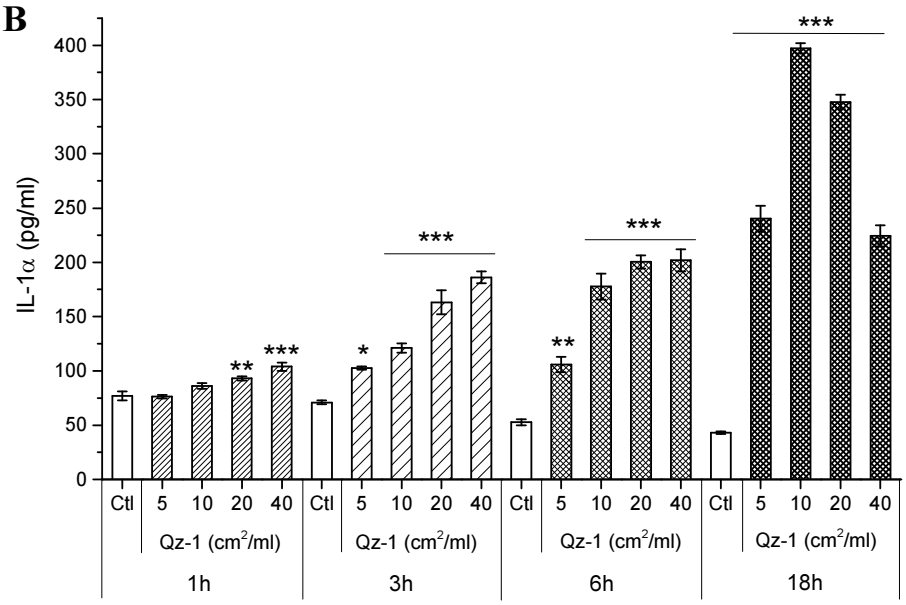

Fig. 4.1 Kinetics of IL-1α gene expression and intracellular IL-1α contents in J774 macrophages. Cells were incubated with increasing concentrations (5, 10, 20, 40 cm^2/ml) of quartz for 1, 3, 6 and 18h. (A) IL-1α expression in macrophages was quantified by qRT-PCR and (B) intracellular levels of IL-1α by ELISA. Determinations were performed in quadruplicate and expressed as the mean ± SEM. Data from a cellular duplicate (A) or one representative experiment out of three (B) are depicted. *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$ *vs* control not exposed to particles (Ctl) in each group.

IL-1α is lately released from necrotic macrophages losing membrane integrity

IL-1α release and cell viability were assessed after incubating J774 macrophages with increasing concentrations of Qz-1 at different time points. As shown in Fig. 4.2A, after 1h and 3h of incubation, no significant release of IL-1α was noted (except for the highest dose of quartz at 3h). The same effect was observed after 6h of incubation, with a significant release of IL-1α only for the 40 cm^2/ml dose. After 18h of incubation we found out an increased release of IL-1α in function of quartz concentration. Cell viability was also evaluated by using the same exposure conditions with two different assays: by measuring the leakage of LDH, a soluble cytosolic enzyme protein that is released into the extracellular medium following loss of membrane integrity [37], and by assessing cell metabolism, in particular the mitochondrial activity, by bioreduction of the WST-1 reagent [38]. Fig. 4.2B shows that cell membrane integrity was lost only after 18h of incubation with increasing quartz concentrations, even if a significant reduction was

observed after 3h with the 40 m^2/ml dose of quartz (experiments at 6h were not performed). Thus, LDH leakage due to plasma membrane damage seems to well correlate with the IL-1α released, although the molecular weight (MW) of LDH (140 kDa) is higher than the MW of pro- and mature IL-1α (31 kDa and 17 kDa, respectively), and likely IL-1α will leak more easily than LDH. Conversely, the WST-1 assay (Fig. 4.2C) did not provide information of interest regarding the mechanism of IL-1α release, as a significant dose-dependent decrease of cell viability was observed already after 1h of incubation with quartz.

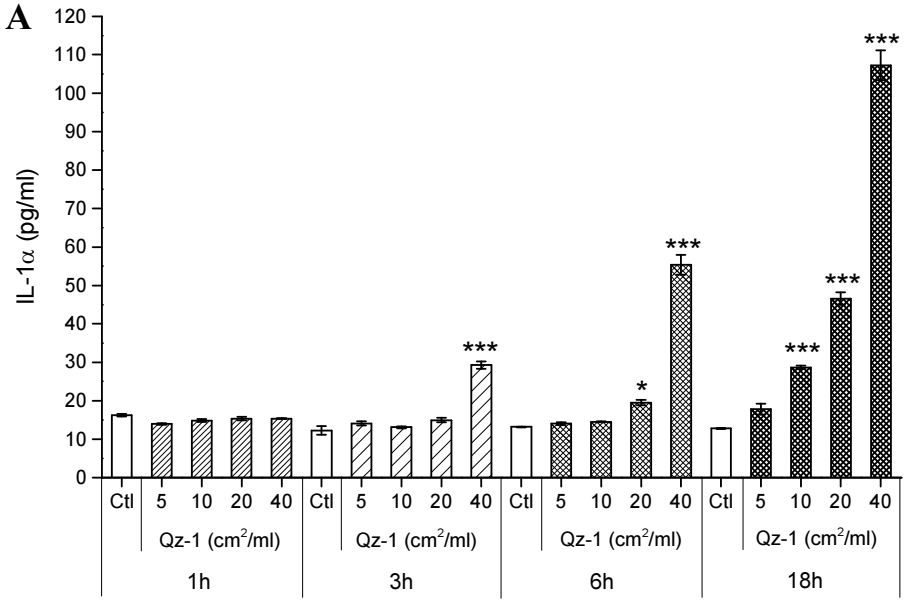

Fig. 4.2 (See legend on next page)

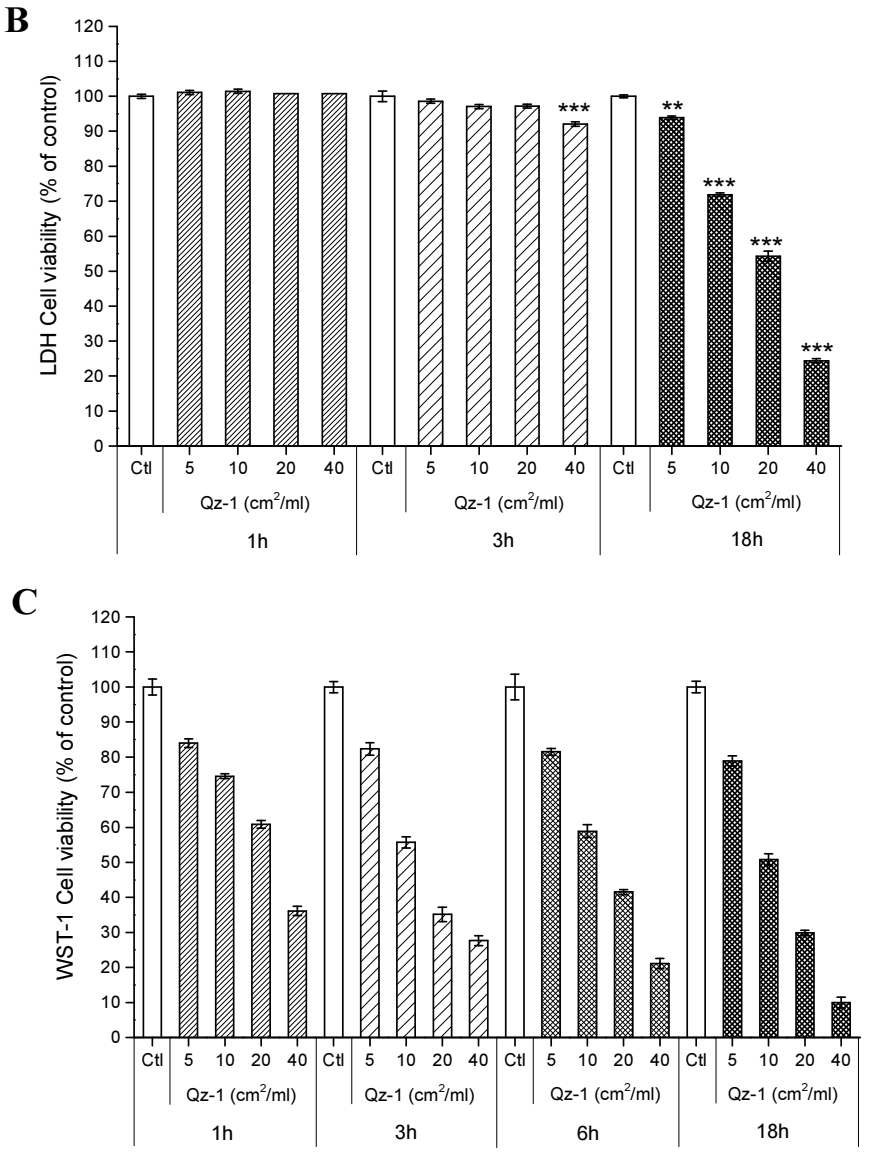

Fig. 4.2 Kinetics of IL-1α release from J774 macrophages and cell viability after quartz exposure. Cells were incubated with increasing concentrations (5, 10, 20, 40 cm^2/ml) of quartz for 1, 3, 6 and 18h. IL-1α released (pg/ml) into culture supernatants was assessed by ELISA (A) and cell viability by LDH leakage (B) and WST-1 assay (C). Results of cell viability are expressed as percentage of the control (macrophages not exposed to silica particles -Ctl). Determinations were performed in quadruplicate and expressed as the mean ± SEM. Data from one representative experiment out of three (A, C) or one single experiment (B) are depicted. *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$ vs control in each group. (C) All exposure concentrations of the particles were statistically different (***$p < 0.001$) from the control.

Varying IL-1α release and cytotoxic activity in response to different silica particles

IL-1α release induced by a set of silica particles with diverse physicochemical properties was measured after 18h of incubation of J774 macrophages with the particles at increasing concentrations. The set of particles is listed in Table 4.1 and comprised quartz dusts characterized by different surface properties and biological activity (Qz-1, Qz-2, Qz-P) [31, 34, 36], synthetic quartz crystals known to be inert in several biological responses (n-Qz-syn) [30], and three amorphous specimens: colloidal silica spheres (MSS) previously used as negative control because of their biological inertness [36], a fumed silica (A50) known to be very active in some cellular tests [33, 39] and finally a vitreous silica (VS) with characteristics similar to quartz dusts except crystallinity [36]. Fig. 4.3A shows the release of IL-1α for the different silica particles at a fixed concentration (40 cm^2/ml) and a large variation in the capacity to stimulate the release of IL-1α depending on particle type was found.

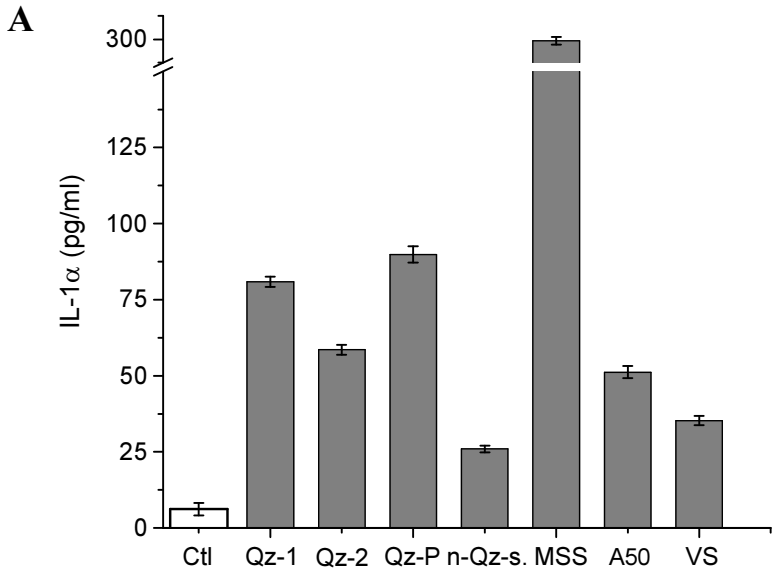

Fig. 4.3 (See legend on next page)

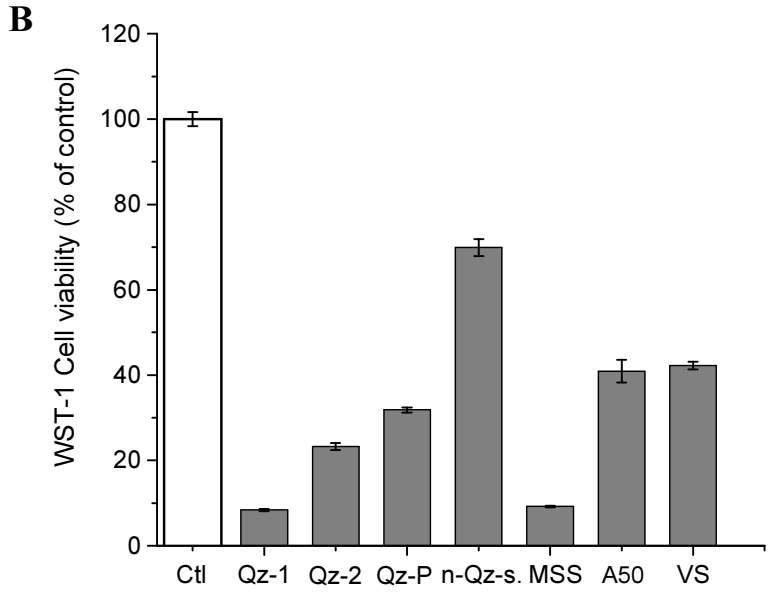

Fig. 4.3 IL-1α release from J774 macrophages and cell viability in response to different silica particles. Cells were incubated with 40 cm^2/ml of silica for 18h and IL-1α released (pg/ml) into culture supernatants (A) and cell viability (B) were evaluated by means of ELISA and of the WST-1 assay. The silica samples included a commercial quartz (Qz-1), the same quartz heated at 800°C (Qz-2), a pure quartz (Qz-P), synthetic quartz crystals (n-Qz-syn), amorphous silica spheres (MSS), a fumed silica (A50) and a vitreous silica (VS). Results of cell viability are expressed as percentage of the control not exposed to particles (Ctl). Values are mean ± SEM from one single experiment performed in quadruplicate. All the silica samples were statistically different (***$p < 0.001$) from the control.

Significant levels of IL-1α were induced by all the silica particles, in a dose-dependent fashion (Fig 4.4). n-Qz-syn was the least active, followed by VS and A50. The three quartz dusts (Qz-1, Qz-2 and Qz-P) situated at an intermediate level of IL-1α induction, while the IL-1α release induced by MSS was the highest (three times greater than the others). The measurement of cell viability by the WST-1 assay (Fig. 4.3B) showed that, at these experimental conditions, all particles were significantly cytotoxic to variable extents, approximately reflecting IL-1α release.

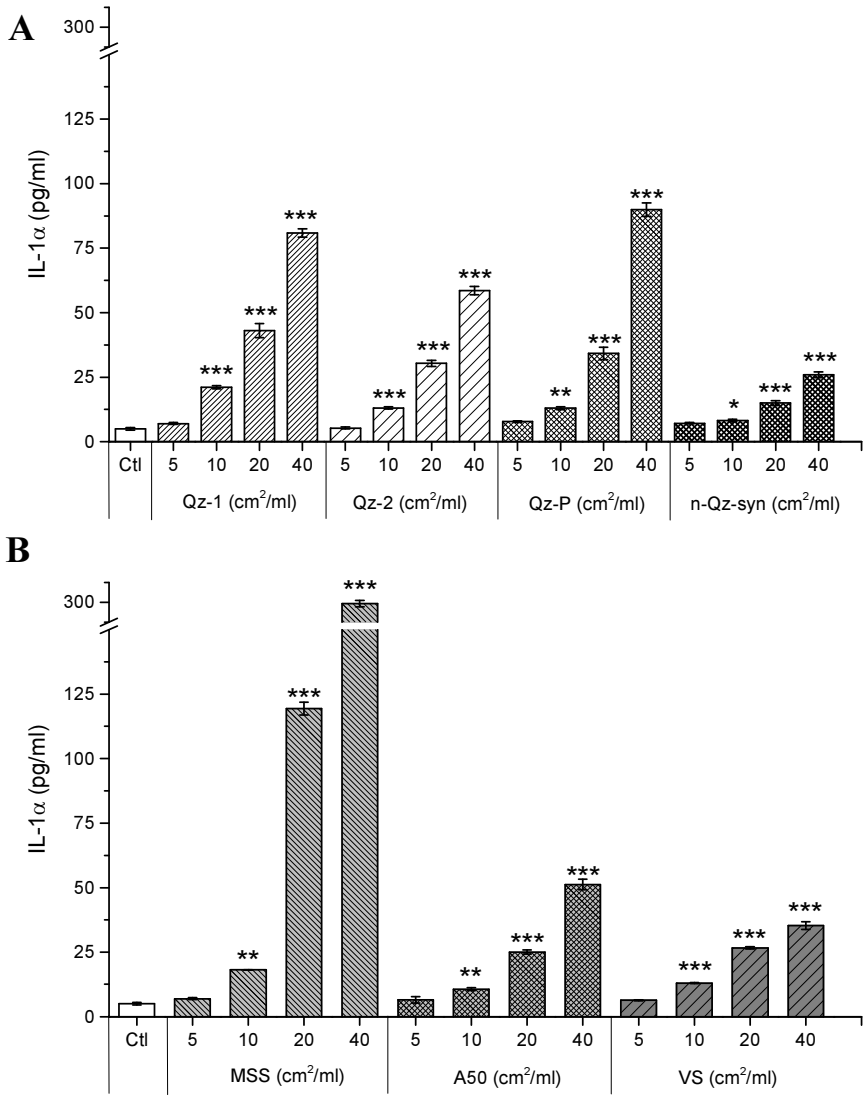

Fig. 4.4 IL-1α release from J774 macrophages in response to increasing concentrations of different silica particles. Cells were incubated with increasing concentrations (5, 10, 20, 40 cm^2/ml) of quartz particles (A) or amorphous particles (B) for 18h and IL-1α released (pg/ml) into culture supernatants was assessed by ELISA. Values are mean ± SEM from one single experiment performed in quadruplicate. *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$ *vs* control not exposed to particles (Ctl).

Release of IL-1α induced by the different silica samples does not correlate with hemolytic activity

In Fig.4.5 the IL-1α levels induced by the set of silica particles under study were reported as a function of their hemolytic activity. The data for hemolysis were obtained from previous experiments reported in [30, 31] or were verified/determined on purpose. No correlation between the hemolytic activity and the IL-1α released was evidenced.

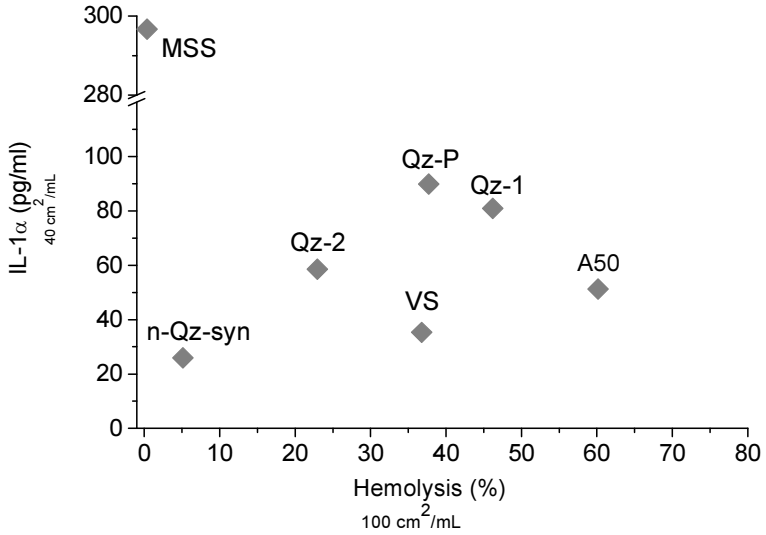

Fig. 4.5 Absence of correlation between the hemolytic activity and the IL-1α release induced by different types of silica particles. Percent of hemolysis at silica concentration of 100 cm^2/ml and release of IL-1α (pg/ml) from J774 murine macrophages at silica concentration of 40 cm^2/ml were compared.

4.3.4. Discussion

The pivotal role of IL-1α as an alarmin and initiator of sterile inflammation [6, 11, 19, 40] has also been noticed in the acute lung inflammation induced by exposure to silica particles. Indeed, upon particle exposure, IL-1α released by alveolar macrophages was found to mediate directly neutrophilic inflammation and to regulate the expression of the IL-1β precursor [26].

The results of the present research point out that *in vitro* the release of IL-1α by a macrophage cell line in response to quartz particles is mediated by two steps:

I) an early event that occurs in a very short time (already after 1h), consisting in the induction of the gene transcription of IL-1α with subsequent production and intracellular accumulation of the protein;

II) a late event, associated with the typical role of alarmin, characterized by the release of IL-1α into the extracellular medium as a result of plasma membrane rupture.

Relying on the finding that, already 1h after silica administration *in vivo* IL-1α protein levels were significantly increased in the bronchoalveolar lavage fluid (BALF) of mice, it has been previously postulated [26] that IL-1α in pre-existing stocks was quickly released by necrotic alveolar macrophages acting as an alarmin. Here we show that *in vitro* IL-1α release occurred late and the results indicate that the mechanism of release is more complex and finely regulated than the mere release of constitutive IL-1α following necrosis. Concerning the delayed *in vitro* release of IL-1α compared to *in vivo*, we have to consider that the J774 macrophage cell line is purely a cellular model incompetent for the inflammasome, the enzymatic machinery involved in the release of active IL-1β and suggested to take part in the release of mature IL-1α [25, 41, 42]. Even though a sole contribution of the inflammasome may be excluded for particles [24, 43], inflammasome activated-caspase-1 was found to facilitate the secretion of IL-1α by cleaving the IL-1R2 receptor which inactivates the enzyme responsible for IL-1α maturation, calpain [44]. Moreover, *in vivo* some other cell types, such as lymphocytes, dendritic cells, epithelial and endothelial cells may be implicated in the prompt release of IL-1α [26, 45, 46].

The current finding opens additional pathways still to probe on the regulation and release of the alarmin IL-1α. With step (I) we pointed out that IL-1α is present in J774 macrophages not only as pre-existing stocks, but it is produced in a very short time following a signal of cell stress as shown with WST-1 data (see Fig. 4.1 and 4.2C). The macrophage cell is therefore able to detect silica particles very quickly (less than 1h) when it is possible that particles are not yet internalized. We can then speculate on several pathways of activation of IL-1α gene transcription: 1. an indirect activation through mediators (e.g. ROS), 2. activation of outer cell membrane receptors (not Toll-like receptors) recognizing specific functionalities present on silica, 3. activation as a result of particle internalization.

With step (II), the release of IL-1α from necrotic cells undergoing swelling of the cytoplasm and plasma membrane damage was noted. This latter finding presupposes a passive mechanism of IL-1α release, even if an earlier active secretion of the mature form is not to be excluded. Indeed, preliminary western blot experiments (not shown here as the protocol for IL-1α determination should be improved) showed the presence of the mature form of IL-1α (17 kDa) into culture supernatant, suggesting an active mechanism of IL-1α secretion.

One further evidence that IL-1α release is not based only on a membranolytic mechanism is the absence of a correlation with the ultimate

test on membrane damage, the hemolysis test, for a set of silica particles characterized by different surface and bulk features. For some of the investigated particles there are signs of correlation, such as the decrease of the release of IL-1α (as previously observed for IL-1β [32]) and of the hemolytic effect [31] upon heating Qz-1 at high temperatures, reducing surface hydrophilicity [28, 47]. A low release of IL-1α was observed for n-Qz-syn which is known to be inactive in several endpoints of cellular toxicity, including hemolysis [30]. However, some other samples that were highly active in RBC membrane rupture, i.e. A50 and VS [31] induced a moderate release of IL-1α. Conversely and surprisingly, the MSS silica particle that has always been found inert in all *in vitro* toxicological assays [33, 36], here it was the sample inducing more IL-1α release. These results suggest that the mechanism of IL-1α release by J774 macrophages is not merely based on membranolysis. Indeed, it is known that MSS does not cause LDH leakage [33, 36], although here induced high metabolic stress (WST-1 assay). MSS is a peculiar silica particle highly hydrophobic and composed by regular round spheres of 1μm diameter [36]. It is possible that due to these unique properties, MSS may interact with membrane proteins in some specific ways (denaturation?), thus causing an early upregulation of IL-1a expression not previously found with other cell tests measuring some other toxicity endpoints. Further studies are needed to check the membranolytic ability (LDH leakage) of the present set of particles and their capacity to induce the genetic transcription of IL-1α, especially in alveolar macrophages which may react differently from J774 cells.

In conclusion, contrary to IL-1β secretion which is well correlated with hemolysis and is dependent on a membranolytic effect (the lysis of the phagolysosome membrane), the release of IL-1α does not correlate with hemolysis. Therefore, this inflammatory marker appears not merely dependent on membranolysis, as also demonstrated by preliminary western blot experiments and by the early induction of IL-1α gene transcription. An additional secretory process, that is not dependent on plasma membrane integrity, appears to be involved. These potentially new pathway of particle mediated-induction of IL-1α gene expression should be explored.

4.3.5. References

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CHAPTER 5

From “model” to new fibrogenic quartz-rich dusts: Is membranolysis predictive of the pathogenicity of complex materials?

5.1. Summary

Context: new outbursts of silicosis were recently reported due to processing of a material known as "artificial stone”. The unusual severity of the observed diseases led to suppose that peculiar physico-chemical features of the dusts generated by cutting artificial stones may drive the adverse effects as, beside high content of quartz, artificial stone can contain resins and pigments which may modify the well-known reactivity of quartz.
Experimental design: a set of artificial stone dusts – collected in Italian companies where some cases of silicosis were reported – were physico-chemically characterized and their potential to generate free radicals in acellular systems, to induce membranolysis, to elicit cytotoxic effects on bronchial epithelial cells and alveolar macrophages, and to induce the epithelial-mesenchymal transition (EMT) in epithelial cells was investigated.
Main achievements contributing to the knowledge on silica toxicity: artificial stone dusts exhibited a strong potential to induce oxidative stress in *cell-free* tests – likely because of the large presence of redox active transition metals – and induced the EMT in epithelial cells, a process which may contribute to explain the development of fibrosis on workers exposed to artificial stone dusts. On the contrary, they resulted inactive in membranolysis and only slight cytotoxic on epithelial cells as for a masking effect due to the presence on the particle surface of a polymeric resin.
For the first time artificial stone dusts were investigated in a toxicological perspective. The present data suggest that some of the classic endpoints used for evaluating quartz toxicity, such as cytotoxicity and hemolytic activity, may not be adequate to describe the biological activity of more complex quartz dusts. These findings confirmed that hemolysis is unrelated to free radical generation, and that particle surface need to be accessible in order to interact with membranes.

5.2. *Appended paper E (submitted to Toxicological Sciences):*

""Artificial stone dusts": a new cause of an ancient disease. Particles generated by abrasion exhibit a radical activity higher than quartz and induce epithelial-mesenchymal transition in human bronchial epithelial cells"

Author's contributions

This research was conducted within the Centro Scansetti, University of Turin in a collaboration between the Dept. of Chemistry and the Dept. of Oncology, in particular with the skillful contribution of MP and DG and was supervised by BF and MT. CP performed the physicochemical characterization of the artificial stone dusts, the measurements of free radicals, TGA analysis at the Dept. of Chemistry, with the essential help of MT, IC, FT and Francesca Ardizzi and carried out the hemolysis tests at the Dept. of Oncology. CP processed these data and wrote the manuscript in collaboration with MP, MT and BF.

CHAPTER 6

General conclusions

Exposure to crystalline silica dusts in respirable size has long been recognized as cause of debilitating pathologies such as silicosis, lung cancer and autoimmune diseases [1]. In particular, silicosis has been reported for many decades as one of the major occupational health issues [2, 3]. In Italy silicosis is recognized as an occupational disease and is compensated by the Italian Worker's Compensation Authority (INAIL) which has always shown a great attention to this occupational disease including funding this PhD.
Prolonged exposure to silica particles may occur in some professional settings (e.g. mining, sandblasting, construction, clothe sanding) and recently new outbreaks of silicosis were reported [4] such as those related to processing of artificial stones [5], reporting on the stage one of the most ancient professional pathologies.
However, not all silica sources were found equally pathogenic [6] and differences from one to the other silica source were often reflected in experimental studies [7]. The large variability in the hazard associated to the exposure to both quartz particles [8] and amorphous nanosilicas [9] stems from two main factors as follows:

i. the chemical nature of the dust. Silica particles which have all the same chemical composition may exhibit different physico-chemical properties and surface functionalities - crystallinity, size, morphology, fresh fractures and hydrophilicity, external contaminants such as metal ions and impurities acquired during processing - which can modify the silica surface [10].
ii. the multiple interactions of silica particles with biomolecules and cells within the respiratory system, each interaction being possibly modulated by different physico-chemical features of the particles [1, 10].

Over time most studies looked for one single property explaining silica toxicity; however, to the current state of the art it is evident that more than one physico-chemical property contributes to the pathogenic potential of silica and that different physico-chemical features are implied in each step of the cascade of the cellular/biomolecular events generated after contact of the silica particle with living matter.

Despite the wealth of studies on the topic, the extent to which a given surface property governs a specific adverse biological response and the cellular and molecular events leading to inflammation and fibrosis at the origin of silicosis are not still completely clear, mostly because of the intrinsic complexity of the cellular mechanisms involved and of the high heterogeneity of the silica surface. A major contribution to the mechanisms of silica toxicity has been given by the discovery of the multi-enzymatic complex inflammasome which mediates the inflammatory response induced by silica particles [11]. However, no results on how the physico-chemical property of silica modulated this essential machinery were available at the beginning of this research.

In this thesis the variability of silica toxicity was addressed trying to simplify the complex interaction silica surface-biological matter by considering one of the starting events triggering the first cellular steps contributing to silica toxicity [12]. This key promoter event is membranolysis and RBCs were used as model to probe silica membranolytic activity along all the research. Moreover, to unravel complexity associated to silica dusts two strategies were adopted which consisted in: i) the use of model silica particulates (such as tailored synthetic quartz crystals or commercial quartz and amorphous silicas of controlled surface properties, modulated by specific physico-chemical treatments) or ii) combining a large number of carefully selected silica particles in a single research work and comparing the chemical/biological outputs relying on a physicochemical property of interest.
Such research has been conducted with the final goal to shed light on the silica properties responsible of the toxic effects. Making advantage of the relationship between physico-chemical reactivity and biological effects, we have identified one or more features that can be analysed in order to predict the pathogenic potential of a given source of silica. To achieve this main goal the role of silica surface reactivity in membranolysis and activation of inflammatory pathways was addressed. Some minor objectives were set during the study, which are indicated in the Scheme 2 of the Chap.1 (pag. 18). As already indicated in the Summary section of each Chapter, the main achievements reached during the course of this thesis are hereafter schematically summarized.

- Among the ***physico-chemical properties of silica particles*** which may be ***relevant to membranolysis*** we found that a key role is played by the surface distribution of silanol, silanolate and siloxane functionalities. In particular, membranolysis appears not simply related to silanol density but rather to a specific surface distribution of dissociated/undissociated

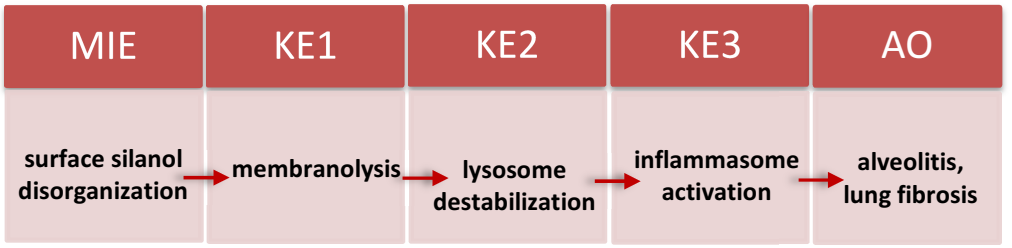
silanols and siloxane groups, as silica particles with intermediate surface hydration were the most hemolytic ones. Some other physico-chemical properties, essential in the pathogenic response to silica, such as crystallinity and ability to generate free radicals resulted not involved in silica hemolytic activity (*Appended paper A*).

- According to the previous results, we focused on the silica features found relevant in inducing the membranolytic effect to better understand ***how these surface functionalities modulate membranolysis.*** By investigating three different aspects concerning the surface features of silica, i.e. (i) fracturing (*Appended paper B*) of intact as-grown crystalline surfaces (*Appended paper F*), (ii) the distribution of acidic surface sites (*Appended paper C*) and (iii) different surface silanol patterns (Chap. 3), it resulted that a silica surface characterized by disordered arrays of silanols and siloxanes, hence by a high heterogeneity of the surface functionalities (imparted by fracturing, by the presence of framework impurities, by abrogating mutually H-bonded silanols), is the most membranolytic (and the most reactive and cytotoxic) one.

- We next focused on the ***role of membranolysis in the inflammatory response induced by silica*** as inflammation is the first step leading to silica pathogenicity and in order to clarify how RBCs, even being a cell type not involved, may nevertheless be relevant for the mechanism of silica toxicity if used as model of cell membranes. The *in vitro* investigation of some key and emergent inflammatory pathways (the release of the cytokines IL-1β and IL-1α) led to correlate the RBC membrane rupture with the destabilization of the phagolysosomal membrane subsequent to particle phagocytosis by alveolar macrophages (*Appended paper D*). On the contrary, the release of IL-1α did not correlate with hemolysis, probably because the mechanism of IL-1α release is more complex and finely regulated than simply plasma membrane injury (Chap. 4).

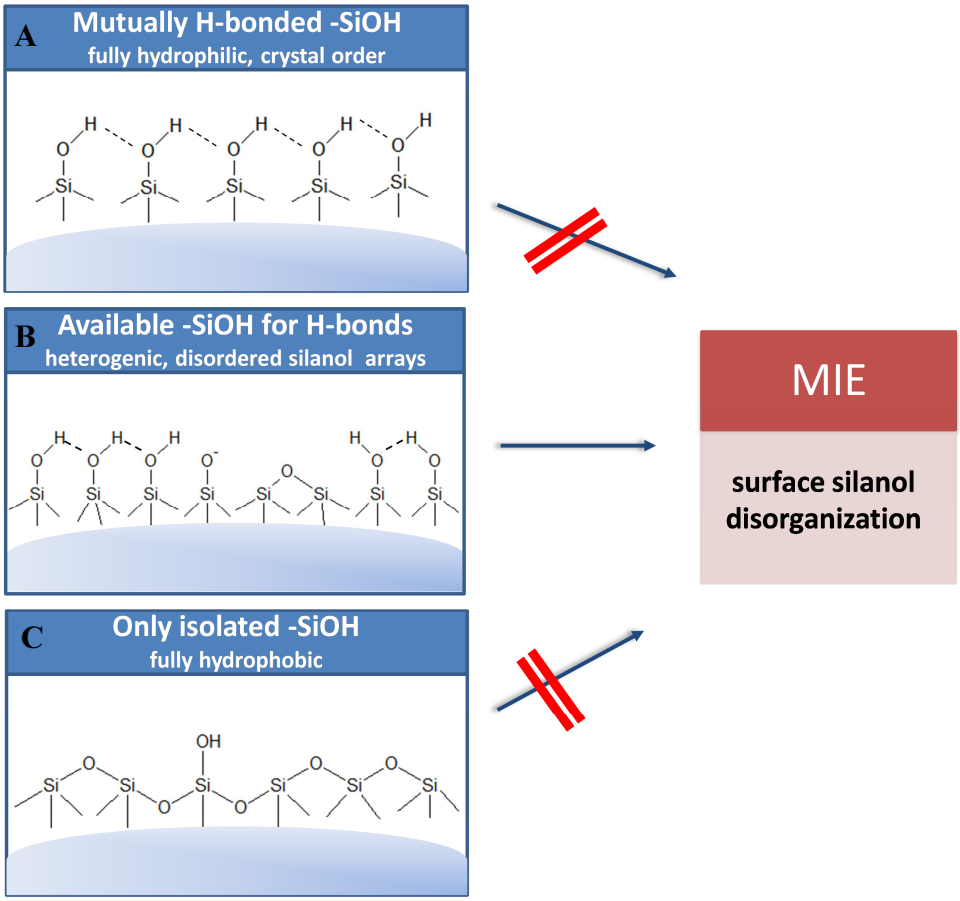
Based on the whole achievements obtained from this study we propose here an Adverse Outcome Pathway (AOP) [13] concerning the fibrogenic activity of silica in the lung (Scheme 1). For the first time in the study of silica toxicity, the heterogeneity of the surface functionalities of silica, thus the surface silanol disorganization, is advanced as the Molecular Initiating Event (MIE) leading to the adverse outcome (AO) through a number of key events (KE). The events that in this thesis are recognized as essential for the development of lung fibrosis are cell membrane damage (KE1) which is

associated to the destabilization of the lysosome membrane in alveolar macrophages and release of the lysosome content into the cytosol (KE2). This latter event triggers the activation of the inflammasome machinery (KE3), starting an inflammatory response that ultimately affects fibroblast activation and proliferation [14] which contribute to the development of silicotic nodules and fibrosis.



Scheme 1 Simplified AOP for silica and lung fibrosis.

We thus identified the surface silanol disorganization as the MIE and further tried to understand the type of chemical bond/van der Waals interaction established between the silica surface and the membrane which is the cause of membrane damage. Contrary to most of the metal oxides, the Si-O-H functionalities of silica (i.e. silanols) never release hydroxyl anions but are strong H-bond donor and acceptor sites with possible H-bonding between them or with other molecules [15].
As resulted from this study (Scheme 2), when most silanols are mutually H-bonded - on real crystal planes and highly hydroxylated surfaces - the silica surface is unreactive towards membranes (Scheme 2 case A). The same happens on highly dehydrated surfaces where few isolated silanols are present on most siloxane exposed surface (Scheme 2 case C). In both these situations no strong H-bond can be established with other molecules. In the first case because the long chains of interacting H-bonded silanols are stabilized by delocalised charges and would require a very high energy to interrupt the bonding among silanols in order to establish new ones [16]. A new scenario is given by the intermediated situation in which a large variety of free silanols (e.g. geminals, terminals, vicinals, isolated) establish particular arrays of strong H-bonding points on the surface, each one oriented in a specific direction (Scheme 2 case B). On such patterns some membrane components may strongly be attached onto the particle surface, such as phosphate ester groups of phospholipids or secondary amide groups or nitrogen/oxygen in an amide of membrane proteins [12, 17].



Scheme 2 Chemical bases of the proposed MIE.

Finally, the dusts obtained by processing the artificial stone, a more complex material rich in quartz but also containing polymers and a large amount of metals, was approached. All samples generated a remarkable amount of active free radicals. The solid system being more complex, the biological responses - such as the membranolytic activity - were affected by the presence of the organic residues which exerted a sort of masking effect on the surface of the particles. By removing the polymeric residues, the particles acquired membranolytic and cytotoxic potential. Interestingly, the epithelial mesenchymal transition (EMT) was induced by both masked and unmasked particles, possibly suggesting in this case a more relevant role of ROS largely produced by artificial stone dusts.

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Appendix: Appended papers

A. In search of the chemical basis of the hemolytic potential of silicas

B. The pathogenicity of silica: crystallinity and surface disorder. Revisiting the paradigm with synthetic quartz crystals

C. Z potential as a tool to evidence impurities and heterogeneity in silanol acidity at the surface of quartz

D. Why does the hemolytic activity of silica predict its pro-inflammatory activity?

E. ""Artificial stone dusts": a new cause of an ancient disease. Particles generated by abrasion exhibit a radical activity higher than quartz and induce epithelial-mesenchymal transition in human bronchial epithelial cells

F. Synthesis of α-quartz with controlled properties for the investigation of the molecular determinants in silica toxicology