The effect of aluminium and sodium impurities on the in vitro toxicity and pro-inflammatory potential of cristobalite

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\section*{ABSTRACT}

\textbf{Background:} Exposure to crystalline silica (SiO$_2$), in the form of quartz, tridymite or cristobalite, can cause respiratory diseases, such as silicosis. However, the observed toxicity and pathogenicity of crystalline silica is highly variable. This has been attributed to a number of inherent and external factors, including the presence of impurities. In cristobalite-rich dusts, substitutions of aluminium (Al) for silicon (Si) in the cristobalite structure, and impurities occluding the silica surface, have been hypothesised to decrease its toxicity. This hypothesis is tested here through the characterisation and in vitro toxicological study of synthesised cristobalite with incremental amounts of Al and sodium (Na) dopants.

\textbf{Methods:} Samples of synthetic cristobalite with incremental amounts of Al and Na impurities, and tridymite, were produced through heating of a silica sol-gel. Samples were characterised for mineralogy, cristobalite purity and abundance, particle size, surface area and surface charge. In vitro assays assessed the ability of the samples to induce cytotoxicity and TNF-$\alpha$ production in J774 macrophages, and haemolysis of red blood cells.

\textbf{Results:} Al-only doped or Al+Na co-doped cristobalite contained between 1 and 4 oxide wt\% Al and Na within its structure. Co-doped samples also contained Al- and Na-rich phases, such as albite. Doping reduced cytotoxicity to J774 macrophages and haemolytic capacity compared to non-doped samples. Al-only doping was more effective at decreasing cristobalite reactivity than Al+Na co-doping. The reduction in the reactivity of cristobalite is attributed to both structural impurities and a lower abundance of cristobalite silica in doped samples. Neither non-doped nor doped crystalline silica induced production of the pro-inflammatory cytokine TNF-$\alpha$ in J774 macrophages.

\textbf{Conclusions:} Impurities can reduce the toxic potential of cristobalite and may help explain the low reactivity of some cristobalite-rich dusts. Whilst further work is required to determine if these effects translate to altered pathogenesis, the results have potential implications for the regulation of crystalline silica exposures.

\section*{1. Introduction}

Crystalline silica, in the form of quartz, tridymite or cristobalite, can cause respiratory diseases when inhaled, such as silicosis (Greenberg et al., 2007; Leung et al., 2012), and is listed as a carcinogen by the International Agency for Research on Cancer (IARC, 1997). However, crystalline silica toxicity has been described as 'a variable entity' (Donaldson and Borm, 1998), as it has variable fibrogenic, mutagenic and carcinogenic potency (Donaldson and Borm, 1998; Meldrum and Howden, 2002; Mossman and Glenn, 2013). The variable toxicity of crystalline silica has been attributed to both the inherent characteristics of the silica particle and external factors that can alter the particle surface reactivity (IARC, 1997), including the presence of impurities (Fubini, 1998).

Quartz is the most common form of crystalline silica, however, exposure to cristobalite is also common in some industries, such as the diatomaceous earth industry where processed samples can contain up to 85 wt\% cristobalite (e.g., Bye et al., 1984; Rafnsson and
Gunnarsdóttir, 1997). In environmental settings, substantial quantities of cristobalite have been found in volcanic ash, such as from the 1980 Mount St Helens, USA eruption (Damby, 2012; Dollberg et al., 1986), and the sustained eruptions of Soufrière Hills Volcano, Montserrat, which produced volcanic ash containing up to 25 wt% cristobalite (Horwell et al., 2014).

The open crystal structure of cristobalite, compared to quartz, readily allows for the incorporation of other elements into the structure through substitutions of cations into the silica tetrahedra (Deer et al., 2013). Analyses of volcanic and diatomaceous earth cristobalite samples, by the authors, have shown that they always contain impurities. In volcanic cristobalite, this is predominantly Al and Na (up to 4 oxide wt % combined) (Horwell et al., 2012). In diatomaceous earth cristobalite, Al, Fe, Na, and Ca have been observed (Nattrass et al., 2015) (up to 13 wt% combined (Nattrass, 2015)). The incorporation of these elements into the crystal structure is likely due to the substitution of Al$^{3+}$ or Fe$^{3+}$ for Si$^{4+}$ and with interstitial Na$^{+}$ or Ca$^{2+}$ to balance the charge (Deer et al., 2013).

As with quartz, toxicology studies have shown that cristobalite can increase gene expression and cytokine release in macrophage and lung epithelium models (Perkins et al., 2012; Barrett et al., 1999), as well as trigger inflamasome activation (Peeters et al., 2014; Damby et al., 2015). However, cristobalite exposure in pottery workers did not appear to cause any adverse effects (Cherry et al., 1998), cristobalite-rich volcanic ash has low toxicity in vitro (Damby et al., 2016; Wilson et al., 2000) and in vivo (Cullen et al., 2002; Housley et al., 2002) and, clinically, there is currently no evidence of chronic disease from exposure to volcanic ash (Baxter et al., 2014). In diatomaceous earth workers, the evidence is conflicting; a number of epidemiology studies have attributed increased mortality (Checkoway et al., 1993), lung cancer (Rice et al., 2001) and other lung diseases (Park et al., 2002) to cristobalite exposure. This has been supported clinically (Hughes et al., 1998), however, some studies of different cohorts show no significant increase in lung disease (Rafnsson and Gunnarsdóttir, 1997) and opacities dissimilar to those of silicosis (Harber et al., 1998; Vigliani and Mottura, 1948). Toxicology studies have found cristobalite-rich diatomaceous earth to have low cytotoxicity and pro-inflammatory potential (Nattrass et al., 2015; Bye et al., 1984; Ghiazza et al., 2009), and no fibrotic effect on rats, guinea-pigs or dogs (Wagner et al., 1968). That epidemiology and toxicology studies of impure, occupational or naturally-occurring cristobalite generally show a lower toxicity, compared to toxicology studies of pure cristobalite powders, suggests impurities may play a role in altering cristobalite toxicity.

It has been hypothesised that structural substitutions can inhibit the toxicity of cristobalite in a similar way to treating the crystalline silica surface (Horwell et al., 2012; Nattrass et al., 2015). Treatment of a quartz surface with Al lactate or polyvinyl pyridine N-oxide (PVNO) has been shown to dampen its cytotoxicity, haemolytic potential and ability to damage DNA (Duffin et al., 2001; Knaapen et al., 2002; Stone et al., 2004; Nolan et al., 1981). Treatment with Fe has led to inconclusive results, in some cases causing an increase in reactivity, in others no change and in some cases a reduction in toxicity (Cullen et al., 1997; Fubini et al., 1995). In mixed dusts containing crystalline silica, such as volcanic ash or coalmine dust, the presence of other mineral and amorphous phases can occlude the silica particle surface (Donaldson and Borm, 1998; Ghiazza et al., 2009; Horwell et al., 2012; Tourmann and Kauffman, 1994), and may also interact with the silica surface, as clay extracts have been shown to dampen quartz toxicity (Stone et al., 2004).

Due to confounding factors, such as the presence of other mineral particles and variations in particle size and surface area within and among samples, the role of structural substitutions in controlling the toxicity of cristobalite dusts has not yet been determined. It is important to establish whether the structural makeup of cristobalite is capable of influencing its pathogenic potential so that these data can be taken into account in risk assessment, and, eventually, exposure regulations in cristobalite industries. Therefore, the aim of this study was to determine if structural impurities in cristobalite can alter its toxic potential. Rather than comparing a range of natural samples of uncontrolled composition, we instead assessed the in vitro toxicity of synthetic cristobalite samples with structural impurities introduced by systematic doping with incremental amounts of Al, with and without Na.

2. Materials and methods

2.1. Materials

Crystalline silica with and without impurities was produced using a silica sol-gel method adapted from Chao and Lu (2002b). Colloidal silica sol (Ludox TM-40, Sigma Aldrich) was dried at 120 °C for at least 12 h, in its pure form, or with dopants added. Samples were doped with Al and Na nitrates in equivalent concentrations of 1, 2 and 3 wt% Al$_2$O$_3$ in a 1:1 M ratio of Al to Na, while accounting for the Al and Na already in the silica sol, as measured by X-ray fluorescence (XRF; data in Additional file 1). The concentration range was defined in order to understand the effect of sequentially increasing Al and Na impurity contents, in accordance with abundances observed in natural cristobalite (Horwell et al., 2012). One Al-only doped sample was also produced to constrain the role of Al, where only 3 wt% Al$_2$O$_3$ (and no additional Na) was added to the starting material.

The resultant dried cakes were gently ground by hand and 5 g pressed into 32 mm diameter pellets at a pressure of 120 MPa, using a uniaxial press. Pellets were then heated at 1100 °C in a Carbolite horizontal tube furnace (Durham University; Table 1) at a rate of 4.4 °C/min and held at the target temperature for 24 h, before being cooled at the rate of the furnace. Synthesis experiments could not be conducted in the cristobalite stability field ($> 1470 °C$ (Deer et al., 2013)) because doped samples treated at $> 1300 °C$ melted, due to the decreased eutectic temperature imparted by the impurities. Therefore, a synthesis temperature of 1100 °C was selected as it is partway between the melting temperature and the upper limit of quartz stability (870 °C (Deer et al., 2013)). Further, naturally-occurring and industrial cristobalites are produced metastably at, or below, this temperature in the exposure settings of interest, such as the diatomaceous earth industry and volcanic environments (Horwell et al., 2013; Engh, 2000), and this temperature was appropriate, therefore, from both experimental and exposure perspectives.

<table>
<thead>
<tr>
<th>Sample production conditions (treatment temperature, time, dopant concentrations), and sample mineralogy (XRD and SEM-EDS).</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Sample name</strong></td>
</tr>
<tr>
<td>------------------</td>
</tr>
<tr>
<td>Non-doped</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>Doped 2Al+Na</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>Raw materials SS</td>
</tr>
<tr>
<td>5Al+Na+Na</td>
</tr>
</tbody>
</table>

? represents unidentified phase.

* Phases in parentheses were only observed in trace quantities.
Table 2
Cristobalite abundance, cristobalite primary XRD peak position, and cristobalite chemistry of samples.

<table>
<thead>
<tr>
<th>Samples</th>
<th>Cristobalite abundance (% ± s.d.)</th>
<th>Cristobalite primary peak position (° 2θ ± s.d.)</th>
<th>Cristobalite chemistry (wt% ± s.d.)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>SiO₂</td>
<td>Al₂O₃</td>
<td>Na₂O</td>
</tr>
<tr>
<td>Non-doped</td>
<td>1600_12 92 ± 4</td>
<td>21.95 ± 0.02</td>
<td>99.9 ± 0.4</td>
</tr>
<tr>
<td></td>
<td>1600_4  87 ± 8</td>
<td>21.96 ± 0.04</td>
<td>0.0 ± 0.0</td>
</tr>
<tr>
<td>Doped</td>
<td>3Al+Na  88 ± 9</td>
<td>21.71 ± 0.03</td>
<td>1.4 ± 0.7</td>
</tr>
<tr>
<td></td>
<td>3Al+Na  64 ± 9</td>
<td>21.89 ± 0.04</td>
<td>0.7 ± 0.3</td>
</tr>
<tr>
<td></td>
<td>5Al+Na  63 ± 20</td>
<td>21.87 ± 0.05</td>
<td>0.1 ± 0.1</td>
</tr>
<tr>
<td></td>
<td>3Al    59 ± 3</td>
<td>NA</td>
<td>NA</td>
</tr>
</tbody>
</table>

Cristobalite abundance (peak area by XRD (n = 2)), cristobalite primary XRD peak position (n = 2; indicative of structural impurities), and cristobalite chemistry (EMPA (n > 30)). s.d. = one standard deviation; NA = not analyzed. Raw materials contained no cristobalite so data are not included in the Table.

For comparison, non-doped pellets were also heated for 4 or 12 h at 1600 °C (Table 1), above the tridymite-cristobalite transition temperature (Deer et al., 2013), to produce a non-doped cristobalite with a level of crystallinity more similar to the doped samples (Table 2) and a highly-crystalline non-doped cristobalite, respectively. The starting material (silica sol; SS), dried at 120 °C and ground by hand, with 5 wt % total Al+Na (5A1+Na_SS) and without dopants, were also tested pre-crystallisation, as non-crystalline material controls. Samples and their preparation conditions are presented in Table 1.

A slice of each pellet was mounted as polished resin blocks and coated with 16 nm carbon for analysis by electron microprobe analyzer (EMPA). The rest of each pellet was crushed with an agate pestle and mortar and then ground in a cryogenic mill (to avoid any impact of sample heating on crystallinity) in air at 12 cycles per second for 30 min, to produce a powder. Toxicological assays were conducted on the milled material which was not further chemically digested to isolate the cristobalite, as this is known to alter the crystalline silica surface and reactivity (Fubini, 1998).

2.2. Particle characterisation

The mineralogy of the samples was determined by X-ray diffraction (XRD) using a Bruker AXS D8 ADVANCE with DAVINCI design (Durham University). Sub-samples of the cryogenically-milled pellets were further ground, using an agate pestle and mortar, sieved onto silicon discs to ensure random orientation, and identification of phases was performed using a locked-coupled scan from 2 to 90° 2θ with an 8 s scan time. Mineral identification was performed by comparison with library patterns (ICDD, 2015). Ground samples were also compacted into a deep well mount using the knife-edge of a spatula to ensure random crystal orientation (Batchelder and Cressey, 1998). A run was then performed using a fixed parallel beam (6° 6) and a moving detector to cover the range 18–35° 2θ, allowing the abundance of cristobalite to be determined by peak-area measurements of the primary cristobalite peak (~21.8° 2θ), and accurate measurements of primary cristobalite peak position (an indicator of crystal purity (Damby et al., 2014)) to be obtained.

The chemical composition of cristobalite was measured on cross sections of a sub-sample of each pre-ground pellet by EMPA, using a Cameca SX100 (Ludwig Maximilian University of Munich). A voltage of 15 kV and current of 4 nA was applied to measure Na, Al and Si concentrations. For Fe, Mg, K and S (present in the resin) concentrations, a 40 nA current was used (see Additional file 1). Areas for analysis were selected by the characteristic cracking indicative of cristobalite at room temperature (Carpenter et al., 1998). Backscatter imaging by scanning electron microscopy (SEM) and energy dispersive X-ray spectroscopy (EDS) mapping was also performed, at 15 kV (Hitachi SU-70 FEG SEM, Durham University), to identify chemically-distinct phases in the sectioned pellet.

Particle size distributions were analysed by laser diffraction using a Coulter LS analyser (Durham University), with polarization intensity differentiation scattering (PIDS).Particles were suspended in water until an obscuration value of 8–12% was acquired, and sonicated for 120 s prior to analysis. Data were analysed by Fraunhofer theory and are presented as the cumulative volume %, volume % and median diameter. Particle size was also qualitatively assessed by SEM imaging using a Hitachi SU-70 FEG-SEM with a voltage of 8 kV (Durham University).

Specific surface area was analysed by nitrogen adsorption measurements at 77 K using a TriStar 3000 instrument (Durham University). Samples were dried in nitrogen gas at 120 °C overnight. The Brunauer-Emmett-Teller (BET) theory was applied to measurements at relative pressures of 0.05–0.24, and the results are the mean of three repeated measurements.

Zeta potential measurements were performed to determine particle surface charge, which has been shown to be an important factor in the cytotoxicity of silica nanoparticles to keratinocytes (Park et al., 2013). Samples at 62.5 μg/ml in complete medium (lung medium, containing 10 μl/ml l-glutamine, 10 μl/ml penicillin and streptomycin, and 10% foetal bovine serum, pH 7) were sonicated for 20 min and the zeta potential measured using a Malvern Nanosizer (Heriot Watt University).

2.3. Toxicology

Cytotoxicity of the samples towards J774 macrophages was measured using the alamarBlue® and WST-1 assays (both a measure of metabolic capacity), and the lactate dehydrogenase (LDH; a measure of cell membrane damage) assay. Macrophages were chosen as they are phagocytes and are actively involved in particle clearance from the lung. Toxicity to these cells will result in reduced clearance and, therefore, potential biopersistence of insoluble particles. Further, sub-lethal activation can promote inflammation. Therefore, macrophages play a key role in the physiological and pathophysiological responses to inhaled particles, and use of well-established model cell lines, like J774, allows for testing of this response. Samples were suspended in complete medium and sonicated for 20 min at 37 °C at 50–50 Hz (Ultrawave Q; Heriot Watt University). J774 macrophages were seeded in a 96 well plate (5 × 10⁴ cells per well) and exposed to 100 μl of particle suspension in concentrations of 8, 16, 31, 62, 125, 250, and 500 μg/ml for 24 h or 48 h at 37 °C and 5% CO₂. Following exposure, the supernatant was removed and stored at ~80 °C for LDH and cytokine analysis. For the alamarBlue® assay, a solution of 1 mg/ml alamarBlue® reagent (resazurin sodium salt; Sigma) in saline was diluted 1:10 in complete medium and 100 μl added to the cells. The plate was incubated for 4 h and fluorescence measured with excitation at 560 nm and emission at 590 nm. For the WST-1 assay, cells were exposed to sample treatments in the same way for 24 h, the supernatant was removed from the cells and 100 μl WST-1 (tetrazolium salt; Roche), diluted 1:10 in complete medium, was added to each well. Cells were incubated for 3 h and the
particles in saline were sonicated for 20 min, serially diluted to
lated red blood cells were washed three times with saline and 100
lume of 1 ml of sheep blood in Alsever’s solution (Oxoid Ltd.) was
in
cell membranes, and has been shown to be a good indicator of the pro-
stimulatory cytokines (Mossman and Glenn, 2013; Piguet et al., 1990).
TNF-α production, and levels at the fi rst time point of measured cyto-
toxicity, respectively, using BD™ Cytometric Bead Array cytokine flex
sets (BD Biosciences) according to the manufacturer’s instructions.

Haemolysis was used to measure the ability of particles to rupture
cell membranes, and has been shown to be a good indicator of the pro-
inflammatory potential of crystalline silica (Pavan et al., 2014). A vol-
ume of 1 ml of sheep blood in Alsever’s solution (Oxoid Ltd.) was
centrifuged at 2348 × g for 2 min and the supernatant removed. Iso-
lated red blood cells were washed three times with saline and 100 µl of
cells were added to 3.6 ml saline. Particle suspensions of 1 mg/ml
particles in saline were sonicated for 20 min, serially diluted to fi nal
concentrations of 1000, 500, 250, 125 and 62 µg/ml, and 150 µl of
the particle suspensions added to 96 well plates in triplicate. Next, 75 µl of
the prepared blood was added to each well, the plate covered and
placed on an orbital shaker for 1 h. Post-exposure, the plate was cen-
trifuged at 250 × g for 5 min, 100 µl of the supernatant was transferred
to a new plate and absorbance measured at 540 nm.

In all assays, 0.5% Triton-X in either complete medium or saline was
used as a positive control and untreated cells as a negative control.
DQ12, a quartz sand with high toxic potency and < 5 µm in diameter,
was used as a high-toxicity crystalline silica standard and TiO₂
(Degussa; 25 nm particle size) as a low-toxicity particle standard.

2.4. Statistical analyses

ANOVA general linear model with a Tukey’s post-hoc test was performed
to determine the signifi cance of differences among samples in the
in vitro assays (Minitab 15; * p < 0.05, ** p < 0.01, *** p < 0.001).

3. Results

3.1. Particle characterisation

Cristobalite constituted ~90% of both the non-doped samples he-
ated at 1600 °C (1600_12 and 1600_4) and the doped sample 2Al+Na,
while cristobalite contributed ~60% of samples doped with > 3 oxide
wt% Al or Al + Na (Table 1 and Table 2). The non-doped sample heated
at 1100 °C (1100_24) contained substantial quantities of tridymite
(another crystalline silica polymorph), and cristobalite content could
not be determined due to peak overlap in the XRD patterns (Table 1).
By SEM, the sample appeared to be predominantly tridymite, as it
did not contain the characteristic cracking of cristobalite. Elemental ana-
lysis by electron microprobe on selected cristobalite areas showed that
the Al and Na content in cristobalite increased as the amount of dopant
was increased (Table 2). A shift in the cristobalite primary XRD peak
towards lower 2θ values as dopant concentration increased was ob-
erved (Table 2). This is indicative of increased d-spacing (a measure of
the unit cell size in the crystal), which has been attributed to the pre-
ence of impurities within the crystal structure (Damby et al., 2014).
Albite (NaAlSi₃O₈), a feldspar, was observed in small amounts in the
XRD patterns of 3Al + Na, 5Al + Na, and in trace quantities in 2Al + Na
and 3Al, as these were only detected in long duration XRD scans. Other
Al- and Na-rich crystals, enriched in Si compared to albite but otherwise
similar in chemistry, were observed in all Al + Na doped samples by
SEM-EDS (Fig. 1) but were not detected by XRD and could not be
identified. Albite or Al- and Na-rich minerals were not detected in non-
doped samples (Fig. 1b). Amorphous material constituted the re-
mained of each sample, and amorphous content was greater in doped
samples compared to non-doped samples.

For particles generated for the toxicological assays, by volume %,
median particle diameter was between 5 and 7 µm for milled, doped samples and 1600_4 (Table 3). The other non-doped samples were slightly coarser, on average, with median diameters between 9 and 13 µm. All samples had > 90% particles < 30 µm in diameter (Fig. 2a), and had a bi- or multi-modal distribution (Fig. 2b). By SEM imaging, few particles were observed that were > 5 µm, indicating that, by number %, most particles were finer than the median indicated by laser diffraction (Fig. 3). Samples were sufficiently fine-grained for successful phagocytosis (Fig. 7).

Specific surface area ranged from 1 to 5 m²/g in the synthesised samples, and was at least 19 times higher in the starting materials (Table 3). Surface charge of particles suspended in complete medium was between −9.5 and −11.4 mV for all samples and standards (Table 3).

### 3.2. In vitro toxicology assays

Cell viability was assessed using the alamarBlue®, WST-1 and LDH assays. Cell viability of J774 macrophages, measured by the alamarBlue® assay was sensitive to the positive control DQ12 quartz and not to the negative control TiO₂. A mixture of cytotoxicity results were observed for the non-doped samples, with 1100_24 and 1600_12 demonstrating significant decreases in viability at 24 h at the highest concentration tested (500 µg/ml), while 1600_4 did not. In addition, the cell viability at 48 h was significantly lower than 24 h for both non-doped 1600_4 and 1100_24, suggesting a time-dependent increase in toxicity. In contrast no significant toxicity was observed in the alamarBlue® assay for any doped samples at either time point or at any concentration compared to the control (Fig. 4). A small decrease in cell viability was observed at 48 h compared to 24 h for cells treated with 3Al doped sample (Fig. 4), but this effect is so small it is of little physiological relevance. Leachates and the starting materials (SS and 5Al + Na_SS) did not induce cytotoxicity in the J774 cells according to the alamarBlue® assay (data in Additional file 1).

Cell viability, measured by the WST-1 assay, generated unreliable data, possibly due to particle interference, and so are not presented.

The assessment of membrane damage via the LDH assay was performed after a 24 h exposure. Firstly, the positive control DQ12 induced significant cytotoxicity, while the negative control TiO₂ did not (Fig. 5). All synthesised samples induced a dose-dependent increase in LDH release. All three non-doped samples reached significant LDH release at concentrations of 250 µg/ml and higher (Fig. 5). In addition, the sample doped with 2Al+Na also displayed increased LDH release at exposure concentrations of 250 µg/ml and higher. In contrast, samples doped with 3Al, 3Al+Na or 5Al+Na only reached significant LDH release at exposure concentrations of 500 µg/ml. The data suggest that the 3Al doped sample was the least cytotoxic according to the LDH assay but, due to data variability, the effect of 3Al doped cristobalite was not statistically different from the other doped samples.

For the assessment of pro-inflammatory mediator production, the positive control DQ12 induced a dose-dependent increase in TNF-α production, while the negative control TiO₂ had no significant impact (Fig. 6). For the synthesised, doped and non-doped samples, no significant induction in TNF-α production by treated cells was observed compared to the untreated control at either 4 or 24 h exposure time (Fig. 6).

Light microscopy imaging of treated cells showed potential cellular uptake of particles from all samples, although it is possible particles were adhered to cell surfaces (Fig. 7). Some particles were clearly not engulfed, especially in cells treated with non-doped 1600_12 and 1100_24 samples.

Haemolysis is presented by mass and surface area dose (Fig. 8). As haemolysis is the direct interaction between the cell surface and particle surface it is appropriate to present the data as surface area dose, due to the variable surface area of the samples (Table 3). Firstly, DQ12, the positive control, induced dose-dependent haemolysis, while the negative control TiO₂ had no significant effect. Sample-induced haemolysis was dose-dependent for all samples, except for those doped with 3Al+Na and 3Al, which were non-haemolytic at all concentrations tested (Fig. 8a). Non-doped 1100_24 was the most haemolytic sample, producing a similar dose response to the DQ12 positive control.

By surface area dose, the data do not fall on to one line, suggesting differences in the surface reactivity of the samples. The most potent samples, when expressed as surface area dose, were the synthesised, non-doped 1100_24 and the 1600_12. These were followed by DQ12 and the 2Al+Na doped sample which were almost identical, followed closely by the 5Al+Na doped sample and the non-doped 1600_4. Again, the 3Al+Na and 3Al doped samples did not induce a significant haemolytic effect when expressed as surface area dose (Fig. 8b).

<table>
<thead>
<tr>
<th>Sample</th>
<th>Median particle diameter (µm)</th>
<th>Surface area (m²/g)</th>
<th>Surface charge (mV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Non-doped</td>
<td>1600_4</td>
<td>7.3</td>
<td>4.0</td>
</tr>
<tr>
<td></td>
<td>1600_12</td>
<td>10.9</td>
<td>0.9</td>
</tr>
<tr>
<td></td>
<td>1100_24</td>
<td>8.9</td>
<td>4.0</td>
</tr>
<tr>
<td>Doped</td>
<td>2Al+Na</td>
<td>7.4</td>
<td>2.1</td>
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<td></td>
<td>3Al+Na</td>
<td>6.4</td>
<td>0.0</td>
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<td></td>
<td>5Al+Na</td>
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<td>3Al</td>
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<td>Raw materials</td>
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<td></td>
<td>5Al+Na_SS</td>
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<tr>
<td></td>
<td>DQ12</td>
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</tr>
<tr>
<td></td>
<td>TiO₂</td>
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<td>NA</td>
</tr>
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</table>

Table 3

Physical characteristics and surface charge measurements of samples.
4. Discussion

Chemical impurities have been identified as one potential factor which may affect the variable toxicity of crystalline silica (Donaldson and Borm, 1998). It has been hypothesised that structural Al impurities may have a similar dampening effect on the toxicity of naturally-impure cristobalite, such as that found in volcanic ash and processed diatomaceous earth (Horwell et al., 2012; Nattrass et al., 2015). However, no study, to date, has measured the effect of Al substitutions on cristobalite toxicity, directly. Here, the in vitro toxicity of cristobalite, synthesised to systematically vary concentrations of Al and Na dopants, was evaluated to determine whether chemical impurities can alter reactivity.

4.1. The effect of doping on crystalline silica toxicity in vitro

In general, when treating the J774 cell line in vitro, doping appeared to dampen cytotoxicity of the synthetic samples. This was most apparent in the LDH assay, and supported by the alamarBlue® assay, where only non-doped samples 1100_24 and 1600_12 elicited a significant cytotoxic response. The Al-only (3Al) doping seemed to be the most effective at preventing cytotoxicity in vitro, however, the difference between 3Al and the co-doped (Al+Na) samples was not statistically significant.

The magnitude of cytotoxicity varied by assay. The difference in sensitivity may be due to the nature of the endpoints which include a measure of metabolic capacity (alamarBlue® assay) versus a measure of membrane leakage (LDH assay), suggesting that membrane leakage could be occurring in cells where metabolic capacity is not suppressed. In both assays, the high-toxicity particle control (DQ12) gave a concentration-response relationship, as expected, and the low-toxicity particle control (TiO₂) had low toxicity, as expected, suggesting both assays were performed successfully. Interference of the particles in the

Fig. 3. Representative scanning electron microscopy images of synthetic crystalline silica particles.
WST-1 assay, strengthens the argument for the use of multiple assays to assess cytotoxicity within a study of this type.

The haemolysis results also demonstrated differences among the potencies of the particles. The similarity in potency of the non-doped 1100_24 compared to the DQ12 positive control was clear when expressed as a mass dose, whereas doped samples (also produced at 1100 °C) were more than 28% less haemolytic at the top dose tested. Modifying the dose to the surface area metric suggested that the toxicity of 1600_12 sample, as well as 1100_24, was relatively high compared to DQ12, suggesting a more reactive particle surface than doped samples. In fact, doping with Al only (3Al) eliminated the haemolytic potential altogether, supporting the hypothesis that doping decreased the haemolytic potential of the samples.

Regardless of the magnitude of toxicity, the same trend was observed in all assays: doping generally dampened particle reactivity. The potential mechanisms for this effect are now discussed.

### 4.2. Mechanisms of doping-induced suppression of crystalline silica toxicity

A primary mechanism by which doping likely reduces the toxicity of crystalline silica is by alteration of the particle surface which, in an insoluble particle, interacts with cells directly. Here, doping caused substantial changes in crystal chemistry through structural substitutions, affected crystalline silica abundance, degree of crystallinity and the polymorph formed, all of which can alter the particle surface properties, and, therefore, bioreactivity.

#### 4.2.1. Chemical substitutions and structural impurities

The results of this study indicate that structural impurities in cristobalite reduce cytotoxicity and haemolytic ability. The Al-only doped sample (3Al) was consistently the least reactive (although not statistically different from samples doped with > 3 oxide wt% Al+Na) and, importantly, was the only doped sample (with > 3 oxide wt% dopant) not to have formed accessory minerals such as albite, the presence of which may influence toxicity (discussed below). It also had a similar cristobalite content to Al+Na doped samples. Therefore, we see an empirical threshold of ~1 oxide wt% Al in the cristobalite structure, above which cristobalite cytotoxicity is reduced for these synthetic samples. This same trend was not seen for the haemolytic potential of the samples, where the amount of dopant does not appear to influence the extent of haemolysis in Al+Na.
doped samples, suggesting similar surface chemistry among these samples.

Elements incorporated structurally will be present at the surface of the particle, as well as internally. Al treatment of the quartz surface decreases its cytotoxic potency, ability to induce inflammatory indicators, and its haemolytic potency (Duffin et al., 2001; Nolan et al., 1981). It has been suggested that an Al treatment would bind to silanols (a surface functional group with the connectivity Si–O–H) on the silica surface, replacing H+ and, thereby, prevent the adsorption of cell membrane components to the silica surface (Fubini, 1998). Pavan et al. (Pavan et al., 2013, 2014; Pavan and Fubini, 2016) have shown that the distribution of silanols on the silica surface affects its haemolytic and inflammatory potency, and Turci et al. (2016) have also shown that the silanol distributions on fractured crystal faces are a key factor in
Dosing caused variation in the crystalline silica polymorph produced under the same treatment conditions; the non-doped sample heated to 1100 °C produced predominantly tridymite (1100_24), whereas doped samples treated at that temperature produced cristobalite. This was expected because synthesis was undertaken within the tridymite stability field (Deer et al., 2013), but was considered an important comparison nonetheless. Studies of tridymite toxicity are limited, but its toxic potential has been suggested to be similar to cristobalite, based on their similar structure (Smith, 1998). This hypothesis is supported by Marks et al. (1956), who showed no difference between cristobalite and tridymite toxicity towards guinea pig cells in vitro. However, intratracheally-injected tridymite into rats was shown to be more potent at initiating fibrosis than quartz or cristobalite (King et al., 1953). This suggests that tridymite toxicity is variable, as with quartz and cristobalite, and is in agreement with a study by Mossman and Glenn (2013), which showed no difference, overall, between the pathogenicity of cristobalite and quartz. This suggests that surface properties of the particles are more important than the silica polymorph in determining toxicity.

In the present study, non-doped 1100_24 (tridymite) had a similar cytotoxic capacity to the non-doped cristobalites, and a similar haemolytic capacity to 1600_12 (non-doped cristobalite) based on surface area dose, suggesting similar surface reactivity between the polymorphs. However, by mass, 1100_24 was significantly more haemolytic than either the non-doped or doped cristobalite samples. Lower cristobalite abundance was observed in the doped cristobalite samples (Table 2) compared to non-doped cristobalite. As tridymite was formed at the same treatment conditions as doped cristobalite samples (heated at 1100 °C for 24 h), it is not possible to compare crystal abundance directly between non-doped and doped samples, and it is unclear whether the reduction in crystal abundance in doped cristobalite samples compared to non-doped cristobalite (formed at 1600 °C) is due to the addition of dopants, or differences in production conditions. However, previous studies show that doping with Na (Chao and Lu, 2000) or Al (Chao and Lu, 2002a) can decrease cristobalite abundance, therefore, it is likely that doping resulted in the decreased abundance seen in doped samples here.

It is likely that the lower cristobalite abundance in doped samples contributed to their decreased cytotoxicity compared to non-doped samples. Indeed, the three samples with ~90% cristobalite (1600_4, 1600_12 and 2Al+Na) were more cytotoxic than the three samples containing ~60% cristobalite (3Al+Na, 5Al+Na and 3Al). However, haemolytic potential did not correlate with cristobalite abundance, and the greater reduction in reactivity of the Al-only sample (3Al) in both the haemolysis and LDH assays compared with the >3 oxide wt% Al +Na doped samples, all of which contained ~60% cristobalite, highlights a dependence on additional characteristics beyond crystal abundance, as discussed above.

### 4.3. The effect of doping on particle uptake

Al lactate treatment has been shown to decrease particle uptake by epithelial cells (Schins et al., 2002). The J774 macrophages used in the present study are considered professional phagocytes, and particle

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**Fig. 8. Haemolytic potential of crystalline silica samples to sheep red blood cells.** Haemolysis as a percentage of a positive (Triton X) and untreated control for red blood cells treated with 62–1000 µg/ml sample concentrations for 1 h, presented by a) mass dose, and b) surface area dose. Error bars represent standard error (n = 4). *p < 0.05, **p < 0.01 difference from untreated control.
particle size distributions were similar for most samples, although non-
4.5. Other factors affecting reactivity

Median particle size did not vary much amongst the samples and particle size distributions were similar for most samples, although non-
doped samples were slightly coarser, whereas surface area ranged from 1 to 5 m²/g in the crystallised samples. No correlation between surface area and particle size was observed, possibly due to the presence of pore spaces as, during heat treatment, water and N₂ are removed, potentially leaving pores (Fig. 1). Alternatively, as particle size is measured in solution, it is possible that there was preferential aggregation of some samples compared to others, although all samples were sonicated to try to prevent aggregation. The bi- or multi-model particle size distributions are likely due to the presence of amorphous material, which has a lower hardness and is more easily milled to smaller particle sizes than crystalline material. Differences in surface area or particle size, alone, do not explain differences in observed cytotoxicity or haemolytic potential.

4.6. Implications for the toxicity of natural and industrial cristobalite and regulation

Findings from this study suggest that modification of cristobalite through the introduction of structural impurities, can reduce reactivity in comparison to pure cristobalite. The findings add weight to the hypothesis, posed by Horwell et al. (2012) and Nattrass et al. (2015), that the low reactivity of diatomaceous earth and volcanic cristobalite, in vitro, is controlled by the presence of both ‘inherent characteristics’ and 'external factors', as suggested by the working group of the International Agency for Research on Cancer, in their recognition that crystalline silica can be variably hazardous (IARC, 1997).

Doping with Al, alone, has a relatively clear effect in reducing the potential toxicity of cristobalite, with additional Na impurities appearing to lessen that effect. However, co-doping with Al + Na is more representative of the composition of natural and industrial-related cristobalite, which has never been observed to contain only Al (Horwell et al., 2012; Nattrass et al., 2015).

That crystalline silica has variable toxicity is well established (Donaldson and Borm, 1998). However, regulations, including occupational exposure limits (OELs) or permissible exposure limits (PELs), do not currently take into account this variability. Mostly, a single OEL value is used for quartz and cristobalite although, sometimes, cristobalite is given a lower OEL (i.e. more stringent) than quartz (NIOSH, 2002). However, this is based on outdated understanding, and we propose that the crystal purity and the purity of the dust, itself, in terms of crystalline silica content, are extremely important in determining potential toxicity. Therefore, it may be more appropriate to set OELs based on knowledge of the characteristics of the crystalline silica involved in the occupational exposure.

Further, hazard management responses to cristobalite exposure post volcanic eruption may be impacted by this and future research. During the 1995–2011 Soufrière Hills eruption, communities were frequently exposed to cristobalite-rich volcanic ash (Horwell et al., 2014; Baxter et al., 1999, 2014) in quantities exceeding the American Conference of Governmental Industrial Hygienists’ OELs for cristobalite (Seari et al., 2002). However, a quantitative risk assessment of the likelihood of individuals developing silicosis showed that the ash could be regarded as a ‘mixed dust’ of low to moderate toxicity (Hincks et al., 2006). In future eruptions, we envisage that tailored environmental exposure limits could be set, based upon a more robust knowledge of the structure-toxicity relationship for cristobalite-rich volcanic ash (Horwell et al., 2012) in lieu of clinical studies on silicosis development, which has never yet been documented in volcanic settings (Horwell and Baxter, 2006).

5. Conclusions

Crystalline silica toxicity is variable and it has been hypothesised that inherent and external impurities can both dampen and augment cristobalite toxicity. Here, doping synthetic silica with Al or co-doping with Al and Na, pre-crystallisation, led to an apparent decrease in cristobalite-induced reactivity by Al-only doping was attributed to structurally substituted Al. The decreased toxicity of co-doped samples compared to non-doped samples was also partially attributed to structural Al, however, the decrease in cristobalite toxicity was not able to be explained by Al only. None of the substitutions monitored in vitro are likely to contribute to dampening the toxicity of cristobalite-rich mixed-phase dusts. These findings have implications for the setting of occupational and environmental exposure limits for crystalline silica, which currently do not account for inherent characteristics which may render cristobalite dusts much less toxic than predicted by laboratory standards.
Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data and materials

The datasets used and/or analysed during the current study available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests.

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Authors’ contributions

CN designed the study, performed physicochemical analyses at Durham University and in vitro toxicity assays at Heriot-Watt University, and drafted the manuscript. CJH conceived and designed the study, performed physicochemical analyses, DB participated in in vitro assays. VS supervised CN. All authors helped draft the manuscript, read and approved the final version.

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Appendix A. Supplementary material

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References

