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Inflammatory mechanism induced by natural and engineered silica particles in human-derived macrophages at low non-cytotoxic doses

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INFLAMMATORY MECHANISM INDUCED BY NATURAL AND ENGINEERED
SILICA PARTICLES IN HUMAN-DERIVED MACROPHAGES AT LOW NON-
CYTOTOXIC DOSES

by

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of the
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Dedicated to my parents, Shanti and Premasekharan, and my dear husband, Ankur Kamthe
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ABBREVIATIONS

ROS: Reactive oxygen species
DFO: Deferoxamine mesylate
DTPA: Diethylene triamine pentaacetic acid
MβCD: Methyl-beta-cyclodextrin
MnP: Manganese (III) tetrakis (N-ethylpyridinium-2-yl) porphyrin
D609: Tricychodecan-9-yl-xanthate
PC-PLC: Phosphatidylcholine-specific phospholipase C
PMA: Phorbol 12-myristate-13-acetate
EDX: Energy-dispersive X-ray spectroscopy
ICP-MS: Inductively coupled-plasma mass spectroscopy
DPPP: Diphenyl-1-pyrenylphosphine
MTT: 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
TEM: Transmission Electron Microscopy
SEM: Scanning Electron Microscopy
IL-1β: Interleukin-1 beta
TNF-α: Tumor necrosis factor-alpha
ABSTRACT

Particle size, phase, and transition metals have all been implicated in natural and engineered silica-induced respiratory effects, as well as cellular interactions. However, efforts to unambiguously determine their role in the pro-inflammatory mechanism induction have been hampered due to the use of inhomogeneous samples, with incomplete characterization and the use of high cytotoxic doses. Here, engineered micro- and nano-sized silica particles, which are more homogenous in their materials properties and used in a variety of applications, were characterized and compared to natural silica at a realistic dose level. Natural (2 μm) and engineered silica particles (2 μm and 50 nm) were characterized and controlled for size, morphology, phase, iron presence, surface area, and aggregation. A novel lipid peroxidation-dependent pro-inflammatory mechanism due to the influence of iron, particle size, and phase was hypothesized for these particles under a low non-cytotoxic dose closer to a realistic exposure regime. It was observed that at a 1 μg/ml low non-cytotoxic dose of silica the presence or addition of iron, reduction of particle size, and crystalline phase of natural silica significantly increased superoxide (O$_2^-$) and hydrogen peroxide (H$_2$O$_2$) production in the macrophages. This increase in O$_2^-$ and H$_2$O$_2$ production, further lead to phosphatidylcholine-specific phospholipase C (PC-PLC) - mediated inflammatory mediator or cytokine production in macrophages via lipid peroxidation and lipid raft disruption (large fraction sub-domains of plasma membrane involved in signal transduction). Addition of an iron chelator abrogated these responses, supporting the role of iron in the hypothesized mechanism. Activation of PC-PLC - induced inflammatory response was determined by using PC-PLC inhibitor, Tricychodecan-9-yl-xanthate, which blocked the inflammatory mediator production. Microscopy studies with cell-particle interaction revealed that particle size also influenced the uptake of silica particles in the macrophages.
mainly via phagocytosis, since binding and activation of membrane receptors and subsequent internalization is strongly dependent on nanoparticle size. Also, a high cytotoxic dose of 100 µg/ml showed macrophage particle overload for both particle sizes, with macrophage damage possibly leading to catastrophic release of inflammatory mediators that could obfuscate study of the normal inflammatory response, emphasizing the need for studies with realistic exposure doses. In summary, this work demonstrated the role of particle size, iron, and phase in a lipid raft dependent-inflammatory mechanism induced by particles at a realistic exposure dose via PC-PLC. It should lead to a better understanding of the mechanism and important parameters for the particle-induced inflammatory response of the lungs, and therefore, control of the respiratory effects caused by real-life exposure to natural and engineered particles.
CHAPTER 1: Introduction

The effect of air pollution particles on mortality and morbidity has increased over the years [1-3]. Epidemiological studies have demonstrated increases in respiratory disorders such as chronic emphysema, pulmonary fibrosis, and other airway diseases due to ambient particulate matter exposure [4-6]. Respirable particulate matter (PM) represents particles of varied size fractions; a larger size fraction between 2.5 and 10 μm (PM$_{2.5}$–PM$_{10}$), a fine particle fraction with diameters less than 2.5 μm (PM$_{2.5}$), and an ultrafine fraction with particles smaller than 100 nm [7]. Within the last 10 years, there has been a growing realization that the adverse health effects associated with PM could be mainly due to ultrafine particles, as a result of studies indicating an increase in pulmonary inflammation, oxidative stress, inflammatory cytokine production, and apoptosis in response to nanoparticles [2, 4, 8]. This has been attributed to their small size, which results in a greater surface area per unit mass, and greater biological exposure to their native surfaces or contaminants such as transition metals and organics carried on them [9].

Furthermore, studies have also demonstrated that the deposition of 20 nm particles is 2.7 times greater than 100 nm particles, and 4.3 times greater than 200 nm particles, with less than 25% clearance of 50- and 100-nm particles during the first 24 hours after inhalation. This is particularly important for susceptible individuals, as it has been observed that patients suffering from asthma and other chronic respiratory disorders show higher deposition efficiencies due to their limited ability of clearance [10].

With the growth of nanotechnology, new nanomaterials, such as silicon dioxide, titanium oxide, single-walled carbon nanotubes (SWCNT), silver, and gold nanoparticles, are being developed for use in various applications due to their enhanced properties [11-12]. For example,
titania and silica nanoparticles have higher thermal stability, chemical stability, and surface area compared to their bulk counterparts [13], and are now widely used in many industrial and biological applications. These engineered nanoparticles are synthesized under controlled conditions, often in the presence of surfactants, and their applications include drug delivery systems, purification technologies, biosensors, and tissue engineering techniques. Their rapid proliferation in medical and technological fields therefore warrants an in-depth study of their possible hazardous or toxic effects.

Furthermore, most manufactured/engineered nanoparticles currently have very limited available toxicity data [14]. Although they are more homogeneous in size, shape, and phase compared to environmental PM (diesel exhaust, silica and silicates), their physiochemical properties depend on the synthesis and processing procedures used in their manufacture, resulting in conflicting reports in the available literature on their health effects. It is therefore paramount to investigate well-controlled and well-characterized nanoparticles, in order to understand the origin and mechanisms of their biological effects, provide insight into possible mitigation strategies, and inform regulatory policy.

1.1 Silica particles

Here, findings are reported on the pro-inflammatory effects of well-controlled and well-characterized crystalline and non-crystalline silica particles, as these particles are a common source of human exposure to both natural and engineered nanoparticles. Silicon (Si) is the second most abundant element on earth next to carbon, and in particles of geological origin it is mainly present as silica which may contain trace amounts of transition metal impurities; and aluminosilicates (containing Al; and Si, K, Fe or Ca) [15-17]. B. De Berardis et al. [18] showed that there is an abundance of free crystalline silica particles in particulate matter in an urban
environment, ranging from 1.6-10.4% of the total particulate matter, with more than 87% of silica particles having a diameter of 2.5 µm and less. The few studies that have evaluated the concentration of silica in air in urban areas under non-occupational settings have reported a range of 0.2 - 10 µg/m³ concentration of silica [18-19]. This range can be due to various reasons such as weather, geographical location, and city. In addition to ambient exposure, inhalation of crystalline silica can occur in an occupational setting, such as during silica milling, mining and sandblasting. The World Health Organization estimates that over one million individuals in the United States are occupationally exposed to silica annually [20]. This exposure has been associated with a severe respiratory disease, silicosis, which is a lung pneumoconiosis characterized by alveolar proteinosis and diffuse fibrosis, resulting in progressively restrictive lung function [21]. Apart from silicosis, exposure to crystalline silica has also been linked to autoimmune diseases, such as scleroderma (systemic sclerosis), rheumatoid arthritis, chronic renal disease, and lung cancer [22].

Silica is also manufactured in amorphous forms of various sizes and porosity, such as fumed silica, silica gel, aerogel, and fused silica; and it can also occur in its amorphous state as a bi-product of power stations and metallurgical processing [23]. Synthetic forms of amorphous silica are mainly used in the manufacture of products such as pharmaceuticals, paints, cosmetics, food additives, and fillers [24]. More recently, non-crystalline, porous, nanoscale silica particles – “mesoporous silica” - are being investigated for drug delivery. This material may contain hundreds of empty channels called mesopores, that are arranged in a honeycomb-like porous structure with high surface area and pore volume, that can be filled with therapeutic and/or diagnostic agents for delivery to target organs via inhalation [25]. Due to this increasing use and production, synthetic amorphous silica is becoming a growing concern as a respiratory hazard.
Although amorphous silica is considered to be less toxic than crystalline silica, \textit{in vitro} and \textit{in vivo} studies on it are still limited and inconclusive, and the current understanding of the toxicity of these particles at a cellular level is not clear. Similar to ambient/natural silica, it is crucial to understand the different physio-chemical parameters and resulting cellular mechanisms that may lead to adverse health effects upon exposure to these particles.

\textbf{1.2 Role of particle parameters in triggering natural crystalline and engineered amorphous silica-induced toxicity, oxidative stress and inflammatory reactions}

Nanoparticle parameters such as particle size, presence of transition metals, surface area, surface charge, aggregation, as well as degree of exposure, can severely affect their mode of action, for example lung inflammation.

\textbf{1.2.1 Particle size, morphology, and surface area}

Several studies have demonstrated that nano or ultrafine particles are more toxic than fine particles [26]. For any given mass of particles, as particle size becomes smaller, the particle number goes up dramatically, and the total particle surface area also increases. High numbers of ultrafine particles are likely to present a substantial problem for macrophage defenses in trying to phagocytose the particles. Experimental studies with natural and engineered silica particulates, titanium oxide, SWCNT, and gold nanoparticles have shown size-dependent (7 nm - 5 \(\mu\)m) biological interactions including cell viability, inflammation, genotoxicity, apoptosis, and necrosis in different types of cells such as A549, Raw 264.7, HeLa, erythrocytes, and keratinocytes [27-31]. In addition, particle shape or morphology can greatly influence cellular uptake. The optimal shape for faster particle internalization, binding, and activation of the cell
membrane was been found to be spherical in comparison to rod shaped particles, since membrane wrapping for rod shaped particles takes more time [32]. This clearly emphasizes that particle size and shape play an important role in particle-induced cellular reactions. Studies have also demonstrated that silica-induced cyto-toxicity can be proportional to the surface area of the particles [33-34]. For TiO₂ or ultrafine carbon, particle surface area dose correlated more closely with lung neutrophil influx responses than either particle mass or particle number as dose metrics [35]. However, the effects of size and surface area are inter-dependent, making it difficult to differentiate the effect of one from the other.

1.2.2 Surface chemistry

The surface reactivity of natural crystalline silica particles has been shown to affect its biological interaction. Possible sources of reactive oxygen species (ROS) have been attributed to both particle-derived and cell-derived reactive oxygen species. Particle based ROS generation could be due to the presence of silanol groups (Si-OH) and the ionized silanol groups (Si-O) on the particle surface that can play a critical role in the interaction between the particle and cell membrane. The Si-OH groups can act as hydrogen donors and form hydrogen bonds with oxygen and nitrogen groups of the lipid membrane. This can cause the cell membrane to rupture and lead to enzyme leakage and tissue injury [36]. Freshly ground silica has been shown to cause more membrane damage and inflammatory response than aged silica [37-38]. The regular arrangement of the silicon-oxygen bond when interrupted due to fracturing of the silica can generate homolytic and heterolytic cleavages. Homolytic cleavage produces Si⁻ and SiO⁻, whereas heterolytic cleavage produces Si+, SiO+. These products are very reactive in nature and tend to recombine among themselves and form reactive bridges, or react with the environment to form ROS at the surface and sub-surface layers. For example, the interaction of silica with water
can produce $\text{H}_2\text{O}_2$, HO•, and O• [39]. This combined with cell-derived ROS, generated due to the presence of ascorbic acid enzymes or lipopolysaccharides, could also be responsible for silica-mediated cellular responses such as ROS production [40]. However, HO•, due to its short half-life, might not be able to trigger the initiation of cytokine production, indicating that there could be a different source for particle-induced inflammation. This possible source could be iron, a common contaminant in silica particles, discussed further in detail in section 1.2.3.

The presence of surface charge on the particles could also influence particle-induced cellular responses and interactions. Bagchi et al. [41] showed that the toxicity of the silica particles is caused by the large amount of positively charged counter ions coating the silica surface layer. Bhattacharjee et al. [42] showed that the surface charge of silica nanoparticles influences their capability to induce intracellular ROS formation and oxidative stress. Surface charge of the particles could also influence the aggregation of the nanoparticles, thus changing the outcome of the particle-induced results. However, there have been studies that have failed to observe any significant effect of particle surface charge on cytotoxicity [43].

### 1.2.3 Transition metals

Transition metals, such as iron and copper, may be found on the surface of quartz particles and can also play an important role in their respiratory toxicity. [44-45]. For engineered nanophases, transition metals are frequently used as catalysts during their synthesis. Over 20 different metals are used in nanoparticle catalyst formulations, but most commercial processes use Fe, Ni, Y, Co, or Mo, alone or in combination [46]. The presence of transition metals on the nanomaterial surface (e.g. Fe on silica or carbon nanotube surface) has been identified as a plausible mechanism through which crystalline silica, asbestos and engineered nanoparticles could catalyze ROS generation via Fenton chemistry [14, 46-48]. This might further lead to the release
of pro-inflammatory cytokines and culminate in fibrosis [40, 49]. This radical generation has been attributed to the Fenton reaction between trace iron and hydrogen peroxide generated during phagocytosis of silica particles by alveolar macrophages. Even in the case of high purity silica, trace amounts of iron could still trigger ROS production and subsequent events. However, the upstream transition metal-cell surface reactions culminating in pro-inflammatory cytokine release has not been definitively determined.

1.2.4 Dosimetry
Studies by Davis et al. [19] and Chow et al. [50] found crystalline silica levels in the particulate matter in 25 US metropolitan cities and the San Joaquin Valley, both urban and rural areas, ranging from 0.2 to 10 μg/m³. To relate the approximations of the ambient levels to that of in vitro studies, Paur et al. [51] used the following parameters: air inhaled/day - 25 m³, concentration of particles in air - 10 μg/m³, lung surface area - 100 cm², and average deposition efficiency of 30%. Based on these parameters, they estimated that for a particle of 100 nm size, with an average surface area of 10 m²/g, the realistic average dose per day would be closer to 7.5 x 10⁻⁴ μg/cm² of the lung surface area and a lifetime dose would be closer to 6.6 μg/cm² in a non-occupational setting for a 100 nm particle. This lifetime dose (80 years) corresponds to the one-time doses (100 μg/ml to 1 mg/ml) that are delivered to cells in the majority of in vitro studies. In other words, typical in vitro studies do not correlate well with realistic exposure scenarios, and may not accurately indicate the particle physiochemical properties and biological response mechanisms relevant under conditions of chronic exposure, where inflammation is likely to play a key role. For example, it is well known that under high exposure levels particle overload can occur and impair the macrophage clearance mechanism as observed by Renwick et al. [52]. Particle-laden macrophages at higher doses were unable to phagocytose all the particles, leading
to impairment in the macrophage phagocytosis mechanism. This could potentially lead to diminished clearance of particles by the macrophages and alter the cellular response observed. Therefore, to study the respiratory effects of nanomaterial particulates, it is essential to perform experiments with low, non-cytotoxic doses that more closely resemble realistic exposure levels.

As inhalation is one of the primary routes through which particles can interact with cells, it is important to study the effect of these particles on lung alveolar macrophages, one of the first defense cells that come in contact with particulates. In this thesis, an interdisciplinary study with equal emphasis on characterizing the described particle parameters and understanding the underlying particle-induced inflammatory mechanism was carried out.

### 1.3 Mechanisms triggering natural crystalline and engineered amorphous silica-induced toxicity, oxidative stress and inflammatory reactions

The exact sequence of events that occur from silica exposure to disease manifestation are unknown. It is largely accepted that alveolar macrophages are a relevant cell type to study, since inhalation is one of the primary routes of entry for silica particles and macrophages are one of the first cells that they come in contact with. The pathogenesis of silica-induced lung disease is mainly initiated with inflammation, i.e. the production of inflammatory cytokines, chemokines, and other growth factors [53-54]. Signaling pathways, such as activation of NF-κB and AP-1, have been identified to mediate the transcriptional control of cytokines and chemokines mRNA [40, 55-56]. However, the upstream events that lead to silica-induced inflammatory cytokine release in macrophages are still inconclusive. Researchers have developed several hypotheses. For example, a study by Hamilton et al. [57] indicated that Macrophage Receptor with Collagenous Structure (MARCO) receptors may be involved in silica uptake and cell death
signaling through activation of protein kinase pathways. However, there are still other types of pattern recognition receptors, such as toll-like receptors, that need to be characterized in order to fully understand this mechanism. Free radical damage leading to disease with inflammation as the initiator is by far one of the most possible mechanisms. Shi et al. [54] hypothesized that the hydroxyl radical could be responsible for the NF-κB activation upon exposure to silica. An *in vitro* study by Liu et. al. [58] demonstrated that cytokine release following silica exposure was mediated through phosphatidylcholine-specific phospholipase C (PC-PLC) in a redox-dependent fashion. However, the level of hydroxyl radical production needed to induce inflammation leading to disease is unlikely without the presence of iron on silica particles at typical exposure levels. Therefore, the role that iron on the surface of natural silica plays in upstream PC-PLC activation and cytokine production is in need of elucidation. Similarly, it is also important to understand if and how engineered silica particles, at non-cytotoxic concentrations, contribute to long-term chronic pro-inflammatory disease processes, since they may also contain iron traces as a result of their synthesis.

### 1.4 Goal

The goal of this work was to determine whether and how particle size, phase, and iron concentration of natural and engineered silica particles, interacting with alveolar macrophages at a low non-cytotoxic dose, cause an increase in toxicity and an enhancement of the respiratory burst, and whether hydrogen peroxide production or lipid raft disruption are directly or indirectly involved in inflammatory mediators production.
CHAPTER 2

The effect of iron on low dose natural silica-induced pro-inflammatory production

2.1 Abstract

Silica inhalation can induce respiratory disease. Iron is suspected of playing an important role in silica-mediated respiratory toxicity, but unambiguously determining its role has been hampered by incomplete characterization, use of high particle doses and lack of understanding of pro-inflammatory mechanisms. In this chapter, a novel hypothesis for the mechanism of silica particle-induced increase in cytokine production was investigated. The role of iron in lipid peroxidation-dependent transcription of cytokines in macrophages by ground natural silica particles at a low non-cytotoxic dose was studied.

Particle size, size distribution, surface area, and phase were determined using electron microscopy, nitrogen adsorption and x-ray diffraction. Iron impurity concentrations before and after acid treatment were determined by energy-dispersive x-ray and inductively coupled plasma-mass spectrosocopies. At a low non-cytotoxic dose (1 µg/ml) of 2 µm silica, the presence of iron significantly increased superoxide (O$_2^-$), lipid peroxidation, lipid raft disruption, and cytokine production in macrophages. Iron chelators, deferoxamine mesylate and diethylenetriaminepentaacetic acid, were found to abrogate O$_2^-$ production and inhibit lipid peroxidation, raft disruption, and cytokine induction. Tricychodecan-9-yl-xanthate, a competitive inhibitor of phosphatidylcholine-specific phospholipase C (PC-PLC), which is an upstream participant in NF-κB activation, and manganese (III) tetrakis (N-ethylpyridinium-2-yl) porphyrin, a superoxide dismutase and catalase mimic, blocked silica-stimulated cytokine
production. A pathway of iron-induced lipid peroxidation disrupting lipid rafts and signaling for the production of cytokines through PC-PLC in silica-exposed macrophages is proposed.

2.2 Background and related work

Exposure to respirable silica particles has been associated with silicosis, lung cancer, and other pulmonary disorders [59]. Thus, it remains important to investigate the mechanisms underlying their health impacts. One of the hypotheses developed for silica-induced toxicity is that iron, found in trace concentrations on naturally occurring silica, enhances cytotoxicity [39, 60]. Incomplete characterization, control of surface chemistry and particle size/shape, which could also influence toxicity, and use of high particle doses, which can mask the effect of particle derived toxicity from overwhelmed macrophage clearance capabilities, however, have led to ambiguities in understanding the silica-induced toxicity mechanism involving iron. Current literature suggests that particle surface reactivity and redox signaling likely orchestrate the inflammatory response of macrophages [40, 61-62]. Macrophages exposed to silica can produce increased inflammatory mediator release [63-64], and particle-surface interactions through receptors can activate signaling events including the respiratory burst [57, 65-66]. Ghio et al. [59] showed that iron on silicates increased oxidant and cytokine generation; however, the mechanism for iron-induced cytokine production was not completely resolved. Superoxide (O$_2^-$) and hydrogen peroxide (H$_2$O$_2$) produced from the phagocyte respiratory burst can initiate iron-mediated lipid peroxidation leading to increased inflammatory mediator production. In previous work by the Forman group [58], it was demonstrated that phosphatidylcholine specific phospholipase C (PC-PLC), an enzyme involved in NF-κB signaling pathway activation and the transcription of cytokines [67], was dependent upon silica-stimulated H$_2$O$_2$ production. The
objective in this work was to determine whether iron-mediated disruption of lipid rafts through lipid peroxidation is involved in the mechanism through which iron on well-characterized silica particles lead to inflammatory mediator production and enhancement of the respiratory burst.

2.3 Materials and methods

2.3.1 Reagents and materials

Chemicals were analytical grade and purchased from Sigma (St. Louis, MO) unless otherwise noted. Diphenyl-1-pyrenylphosphine, DPPP (Invitrogen), Tricyclodecan-9-yl-xanthate, D609 (BIOMOL International), Trizol reagent (Life Technologies), 8-isoprostane enzyme-linked immunoassay, ELISA (Cayman Chemicals), TaqMan reverse transcription reagent and SYBR Green PCR Master Mix (Applied Biosystems) were used. Min-U-Sil 5 silica was purchased from Western Reserve Chemicals. Additional materials used were cholera toxin subunit B (recombinant), Alexa Fluor® 488 conjugate (Invitrogen), Paraformaldehyde (Polysciences), Triton X 100(VWR), 4',6-diamidino-2-phenylindole, dihydrochloride (DAPI) (Invitrogen), Alexa Fluor 568 donkey anti - rabbit IgG (Invitrogen), IL-1β antibody ab2105, and TNF α antibody ab6671 (Abcam). Manganese (III) tetrakis (N-ethylpyridinium-2-yl) porphyrin, MnP (Jaramillo et al. 2009), was provided by Dr. James Crapo, National Jewish Health (Denver, USA); and THP-1 cells, a human acute monocytic leukemia cell line, was obtained from American Type Culture Collection (ATCC).

2.3.2 Silica preparation

Min-U-Sil 5 silica, a proxy for natural silica, was divided into two batches. The first batch was left as received (referred to here as natural silica). The second batch was treated to
remove trace metal impurities (called iron-depleted silica) by heating at 450°C for 48 hours and refluxing in 4 N HCl at 65°C for 4 hours, followed by washing in 0.1 M NaCl for 6 minutes at 1500 rpm, repeated 21 times. The supernatant was decanted, rinsed in 2 N NaOH for 2 hours, centrifuged, neutralized with HCl, and rinsed with water. The resultant silica was rinsed several times with Milli-Q water (Millipore, MA, USA) and dried at 65°C for 24 hours. Size separation was carried out on both batches based on Stoke’s law using centrifugal acceleration, and the supernatant was collected and centrifuged at 15,000 g for 10 minutes to collect ≤ 2 μm, so-called, respirable particles. To obtain iron-repleted silica, iron-depleted silica was doped with 1.0 ml of 1 μM FeCl₃ to 1.0 ml of suspended silica (2 mg silica/ml).

2.3.3 Particle characterization

Particle size, size distribution, morphology, and metal contaminants were measured using a Scanning Electron Microscope (SEM) (FEI Quanta 200 SEM) equipped with a tungsten filament and Energy-dispersive X-ray spectroscopy (EDX) detector with ultrathin window and 132 eV energy-resolution. An Agilent 7500 Inductively Coupled Plasma Mass Spectrometer (ICP-MS) (detection limit for iron: 1.5 μg/L or ppb) was used to quantify trace iron impurities. Crystal structure was characterized by X’Pert PRO PANalytical X-Ray Diffractometer (XRD). The surface area of the particles was determined by the Brunauer-Emmett-Teller (BET) method.

2.3.4 Cell culture

THP-1 cells were cultured in RPMI 1640 (ATCC, CA) supplemented with 10% fetal bovine serum (FBS) (Omega Sci., CA), β-mercaptoethanol, 100 μg/ml penicillin, and 100 μg/ml streptomycin in 5% CO₂ at 37°C. They were then differentiated using a standard method [68-70] into adherent macrophages by treatment with 50 ng/ml phorbol 12-myristate-13-acetate (PMA)
for 3 days. The THP-1 cell line is a human monocytic cell line that can be differentiated to macrophages following stimulation with PMA or 1,25-dihydroxyvitamin D3 (VD3) [71]. In recent years, THP-1 cells have been widely established as an in vitro model for native monocyte-derived macrophages in studies of macrophage involvement in inflammatory disease. For all experiments, tissue culture treated plates were coated with 5% bovine serum albumin in phosphate saline buffer, dried at room temperature and rinsed twice gently with water before use.

2.3.5 3-(4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazoliumbromide (MTT) assay

THP-1 differentiated macrophages were seeded on 12-well tissue culture plates with 3×10⁵ cells/ml serum free media per well. The natural silica particles were added to the wells under final concentrations of 1, 25, 50, and 100 µg/ml. After 22 hours exposure, cell survival was quantified by incubating the cells for 2 hours at 37°C with MTT (5 mg/ml). The resulting colored product was solubilized in MTT solubilization solution (10% Triton-X 100 and 0.1 N HCl in acidic isopropanol). The absorbance was measured at 550 nm and the background absorbance was measured at 690 nm using a microplate reader (Molecular Device Corp., CA). The viability of the treated group was expressed as a percentage of the non-treated control group. To check for silica particle interference, another control group with cells (no particles), similar to the experimental control, was run in parallel, except that after adding MTT solubilization solution the same silica concentrations as the experimental group were added and the absorbance was read.

2.3.6 Conventional microscopic and colorimetric nitroblue tetrazolium (NBT) Assay

Cells (2 x 10⁶/well) were differentiated in a 6-well flat-bottomed tissue culture plate and incubated with 2.5 mM NBT solution, along with warm Kreb’s Ringer Phosphate buffer (KRP),
for 30 minutes. Different silica treatments were carried out. THP-1 differentiated cells (2 x 10^6/well) were incubated with 1 μg/ml of natural silica, iron-depleted silica or iron-repleted silica, with or without 1μM Diethylenetriaminepentaacetic acid (DTPA) for 10 minutes. Cells were also incubated with 400 μM Adenosine Diphosphate (ADP) or PMA; as a negative control, cells were incubated in NBT solution containing PMA and 30 μg/ml Superoxide Dismutase (SOD). In some wells, 100 μM MnP or 20 μM Diphenyleneiodonium sulfate (DPI) or 1 mM Deferoxamine Mesylate (DFO) was added prior to silica addition. After incubation, cells were washed twice with warm 1X phosphate buffer saline (PBS), fixed with 7.4% formaldehyde for 5 minutes and rewashed with PBS. NBT is a yellow, water-soluble salt that forms a purple formazan precipitate when reduced by O_2^-, staining the membrane outer face, and its intensity is dependent on O_2^- production. Respiratory burst activity was measured by evaluating the cells for purple formazan staining (NBT-positive) under a microscope (Olympus CX-40, Olympus, Japan). Cell counts were done with a counting chamber grid on random areas to sample the entire culture plate and avoid user bias. For colorimetric evaluation, cells were washed twice with warm PBS, once with methanol, and then air-dried. All unreacted extracellular NBT was completely removed, and formazan deposited on the cells was dissolved by adding 120 μL of 2 M KOH and then 140 μL of Dimethyl Sulfoxide (DMSO) with gentle shaking for 10 minutes at room temperature. The dissolved NBT solution was transferred to a 96-well plate and absorbance was read at 360 nm.

2.3.7 Diphenyl-1-pyrenylphosphine (DPPP) fluorescence

Lipid peroxidation was estimated by using a fluorescent probe, DPPP. THP-1 differentiated macrophages (100,000 cells/well) attached to a 96 well culture plate were incubated with 5 μM DPPP and dissolved in 0.05% DMSO for 15 minutes in the dark. The wells were washed 2-3
times with 1X PBS to completely remove extracellular DPPP. Cells were treated for 15 minutes separately with 1 µg/ml of natural silica, or iron-depleted silica, and pretreated with 100 µM MnP for 15 minutes and 1 mM DFO for 1 hour. Fluorescence intensity of DPPP oxide (DPPP=O) was measured at an excitation of 351 nm and an emission of 380 nm.

2.3.8 Confocal fluorescence microscopy for lipid raft and cytokine determination

Cells were plated on coverslips at 80,000 cells/ml with 50 ng/ml PMA and at the end of 3 days, were incubated with different silica treatments and methyl-β-cyclodextrin (MβCD) for 15 seconds (for lipid rafts) and 12 hours (for cytokines) and fixed with 4% paraformaldehyde (in PBS) for 15 minutes at room temperature. Briefly, the fixed cells were quenched with 50mM Ammonium Chloride (NH₄Cl) (in PBS) for 5 minutes and permeabilized with 0.1% Triton-100 for 10 minutes. The coverslips were then blocked with 1% bovine serum albumin (BSA) (in PBS) for at least 15 minutes and subsequently immunolabeled with fluorophore-conjugated cholera toxin B (CTx-B) (1:100) (for lipid rafts) and rabbit polyclonal anti IL-1β and TNFα (1:100) (for cytokines) in 1% BSA on top of the cells at 37 °C for 30 minutes in a moisturized chamber. For the cytokines, the cells were also incubated with secondary antibody donkey anti-rabbit 568 (in 1% BSA) (1:100) for 30 minutes at 37°C in a moist chamber. Finally, the cells were incubated with DAPI in PBS for 5 minutes, rinsed with water and mounted with water soluble anti-fade mounting medium (Invitrogen, Carlsbad, CA) on a slide. During the whole procedure, slides were washed with PBS 2–3 times between the treatments. Samples were imaged with a Zeiss LSM 510 Meta NLO confocal/multiphoton imaging system.
2.3.9 Cytokine mRNA assay

The mRNA expression for the cytokines TNF-α and IL-1β was determined with Real-time Polymerase Chain Reaction (RT-PCR). THP-1 differentiated macrophages (2 x 10⁶ cells/well) were differentiated in a 6-well flat-bottomed tissue culture plate, treated with particles at 3 and 6 hours, collected and washed with PBS. Total RNA was extracted using Trizol reagent and treated with DNA-free reagent according to manufacturer’s protocols. DNA-free RNA samples were reverse transcribed using the TaqMan reverse transcription system (Applied Biosystems), and RT-PCR was run with a Cepheid 1.2 RT-PCR machine. Reverse transcription reaction product (5 µl) was added to reaction tubes containing 12.5 µl SYBR Green PCR Master Mix and a primer pair specific for cytokine mRNA; the total PCR sample used was 25 µl. Glyceraldehyde-3-Phosphate Dehydrogenase (GAPDH) was used as an internal control and the primers used were TNF-α sense 5’-GCCTCTGTGCCTTCTTTTGA-3’ and antisense 5’-GCAACCTTTATTTCTCGCCA-3’; IL-1β sense 5’-CGACACATGGGATAACGAGGCTT-3’ and antisense 5’-TCTTTCAACACGCAGGACAGGTA-3’ and GAPDH sense 5’-ACCCCCAATGTATCCGTTGT-3’ and antisense 5’-TACTCCTTGGAGGCCATGT-3’. The comparative ΔΔCt method was used for the relative mRNA quantification. The mean threshold cycle values (Ct) were obtained for the control, and cells treated with silica to check for IL-1β, TNF-α and GAPDH. The differences between the mean Ct for control/treated cells and the reference gene, ΔCt, were calculated. ΔΔCt was defined as the difference of the ΔCt (treated cells) and ΔCt (control). The relative mRNA quantification was calculated using 2⁻ΔΔCt. Threshold cycles (Ct) were selected in the line in which all samples were in logarithmic phase.
2.3.10 Statistical analysis

All data were expressed as the mean ± standard deviation. One-way ANOVA and one-tailed unpaired student's t-test were used for significance testing, using *p < 0.05. For multiple data comparisons, one-way ANOVA was followed up with post-hoc analysis. Data are the means of three experiments, each using three experimental points, with control values subtracted from that of the sample, when compared to natural silica. Data values for all the experiments were normalized with BCA protein assay.

2.4 Results

2.4.1 Involvement of iron in silica-induced superoxide production in THP-1 macrophages at a non-cytotoxic dose

Figures 1 A and B indicate that upon size separation, mean natural particle size was 1.76 ± 0.67 μm and mean iron-depleted particle size was 1.70 ± 0.70 μm. The surface areas for natural and iron-depleted silica particles as measured by BET analysis were found to be 8.844 ± 0.064 m²/g and 8.843 ± 0.057 m²/g, respectively. Iron presence was detected in the EDX spectrum and ICP-MS showed 40 ppb for the natural silica sample, while the EDX spectrum and ICP-MS showed no detectable iron for the iron-depleted silica sample. XRD spectra of these particles (Figure 2) matched α-quartz (International Centre for Diffraction Data file # 00-046-1045). Macrophage viability exposed to natural and iron-depleted silica particles at a low non-cytotoxic dose was confirmed by MTT assay (Figure 3), and the intensity of \( \text{O}_2^- \) production was measured by NBT staining. As shown in Figure 4A, cells exposed to 1 μg/ml of natural silica responded as indicated by darkening of the cells by the formazan staining, while cells exposed to 1 μg/ml iron-depleted silica appeared slightly darker than controls. Addition of SOD to the
media, even though it cannot efficiently penetrate between a cell and the surface to which it binds [75], partially inhibited the production of the purple precipitate, suggesting that superoxide was produced. Furthermore, in a quantitative colorimetric assay in which the formazan was solubilized and measured in a spectrophotometer, exposure of THP-1 differentiated macrophages to 1 µg/ml natural silica significantly increased the NBT reduction (3 fold higher than the control, data not shown) compared to 1 µg/ml iron-depleted silica in cells, and decreased when the silica was treated with the iron chelator DFO (1 mM) and DTPA (1 µM) (Figures 4B, 4C). The addition of iron back to the iron-depleted silica increased O$_2^-$ production. This increase was comparable to 400 µM ADP and 50 ng/ml PMA, known stimuli of the macrophage respiratory burst [76]. There was no influence of the addition of DTPA to the cell media and iron-depleted silica, as it did not decrease the baseline O$_2^-$ levels (4C). Treatment with 100 µM MnP, a small molecular weight compound with both SOD and catalase activities, abrogated NBT reduction. Furthermore, 20 µM DPI, an inhibitor of NADPH oxidase O$_2^-$ production, inhibited NBT reduction (4B).

2.4.2 Lipid peroxide production and lipid raft disruption

Lipid peroxidation was semi-quantitatively determined by using DPPP. DPPP reacts with lipid hydroperoxides but does not react with H$_2$O$_2$ [77]. In figure 5, lipid peroxidation significantly increased upon addition of a non-cytotoxic dose of 1 µg/ml natural silica and iron-repleted silica, in comparison to 1 µg/ml iron-depleted silica. Similarly to O$_2^-$ production, lipid peroxidation decreased in THP-1 cells pretreated with DFO and DTPA, and incorporation of DTPA to media and iron-depleted silica did not affect the baseline production. Lipid raft disintegration was observed in the macrophages by immunohistochemistry. Lipid rafts were detected on the cell surface by using fluorescent derivatives of CTx-B, which specifically binds
to GM1 ganglioside, a marker for the presence of lipid rafts on the cell surface [78]. The control and iron-depleted silica showed a weak concentrated green fluorescence in the center and around the cell surface, while introduction of natural silica or iron-repleted silica markedly increased both the intensity and dispersion of fluorescence in the cells (45% and 36% of cells counted, n = 50), which suggested reorganization of the lipid rafts (Figure 6). This signal was very similar to that observed with cells treated with MβCD, which disrupts lipid rafts by removal of cholesterol from the plasma membrane [79-81]. The addition of the iron chelator DTPA to either natural silica (27%) or iron-depleted silica (22%) showed a fluorescence signal similar to that of the iron-depleted silica alone (26%) or control (19%), indicating that the presence of iron markedly enhanced disruption of lipid rafts.

2.4.3 Lipid raft disruption affects silica induced PC-PLC dependent inflammatory mediator production (IL-1β, TNF-α)

RT-PCR was used to determine mRNA expression of IL-1β and TNF-α, two critical cytokines involved in silica-associated inflammation [58]. Cytokine expression at the protein level was also determined. As shown in figures 7A, 7B and 7D, exposure of THP-1 cells to 1 μg/ml of natural silica significantly increased both the mRNA and protein expression of IL-1β and TNF-α, respectively, in comparison to 1 μg/ml iron-depleted silica and control. Silica particles were observed to be near the cell surface during cytokine production (7D). Addition of iron back to the iron-depleted silica sample exacerbated cytokine production, while treatment of iron-repleted silica with the iron chelators, DFO and DTPA, significantly decreased cytokine production. Again, DTPA addition to media and iron-depleted silica did not produce any change in cytokine production. Pretreatment with 100 μM MnP (which prevented lipid peroxidation)
eliminated silica-induced cytokine production (7C). Pretreatment with 40 µM D609 (PC-PLC inhibitor) for 10 minutes abrogated silica-stimulated cytokine expression (Figure 7C), indicating PC-PLC expression in silica-induced inflammatory mediator generation.

2.5 Discussion and conclusion

The aim of this work was to dissect the mechanism by which well-characterized silica particles-induced inflammatory response at a non-cytotoxic dose so as not to overwhelm the cells. As previous studies in the Forman lab and from other laboratories have demonstrated that part of the signaling process through which macrophage induce cytokine production was stimulated by silica [58, 82-84], this study focused on clarifying some remaining issues. In particular, the role of iron in mediating silica-induced pro-inflammatory signaling, focusing on lipid peroxidation and lipid raft disruption, was investigated. The results support a sequence in which iron on the surface of natural silica markedly enhances the respiratory burst production of $\text{O}_2^-$ and $\text{H}_2\text{O}_2$, lipid peroxidation, and disruption of lipid rafts. At the conclusion of this discussion a scheme summarizing these events that are upstream of PC-PLC activation and cytokine production is presented.

Previous studies have shown that particle size and surface area are two of the major determinants of particle-derived toxicity. Therefore, to control for size, both natural and iron-depleted silica were size examined by SEM, BET, and XRD analyses; which showed them to be of similar dimensions (Figures 1, 2), therefore the difference in silica-induced effects observed between natural and iron-depleted silica does not appear to be attributable to particle size or surface area differences [85]. Additional controls as described in the methods section were used to check the influence/interference of silica particles. Recent studies have shown that particles such as carbon black cause interference in cell viability assays by absorbing dyes in the assays.
and reducing the optical signals that may indicate cell viability or death [86]. No significant changes were observed in MTT absorbance values, indicating that silica particles by themselves caused minimal or no interference (Figure 3). In vitro cell line studies in the past have shown that at much higher concentrations of crystalline Min-U-Sil silica, such as 100 and 150 µg/ml, which are lethal to macrophages and closer to a lifetime of human exposure, the cells produced reactive oxygen species, leading to inflammation [12, 87]. The aim in this study was to use a dose that was closer to the realistic exposure level calculated under section 1.2.4. Consequently, a 1 µg/ml dose (the lowest dose that can obtain a measurable response using current methods) was used, which is a non-cytotoxic concentration and closer to the realistic exposure dose per day compared to other studies. Figures 4, 5, and 7 show that even at a non-cytotoxic concentration of 1 µg/ml, O$_2^-$, lipid hydroperoxides, and cytokines were produced, but were augmented in the presence of iron and decreased upon treatment with the iron chelator DFO and DTPA (DTPA was used in addition to DFO since Fe binds more efficiently to DTPA). When iron was removed from the natural silica, the ability of macrophages to initiate these reactions were much lower. When iron was restored to the iron-depleted silica, lipid peroxidation and cytokine production were increased again. The iron responsible for the enhanced effect of natural silica versus iron-depleted silica was confirmed to be on the particle surface and not the iron present in the media. This was shown by addition of DTPA to the media, which did not produce any difference over the baseline levels of DPPP fluorescence or cytokine production.

The source of O$_2^-$ appeared to have been NADPH oxidase, as treatment with DPI, a non specific but widely used inhibitor of NADPH oxidase, reduced NBT absorbance (Figure 4B). While there are other potential sources of O$_2^-$ in cells and even on their surfaces, the respiratory burst of macrophages has been shown in numerous studies to be the principal source of increased...
O$_2^-$ production upon stimulation [88]. Thus, although use of DPI does allow for the possibility of other sources of O$_2^-$, this is unlikely to have been responsible for the observed NBT reduction.

Although O$_2^-$ production was observed in the macrophages upon exposure to silica, due to its short half-life, in the absence of a compound that reacts with O$_2^-$, such as NBT, dismutation to O$_2$ and H$_2$O$_2$ occurs rapidly. The H$_2$O$_2$ can then readily diffuse into macrophages [89]. The Forman lab has previously shown that H$_2$O$_2$ produced by silica-stimulated macrophages was upstream of the signaling, resulting in both an enhanced macrophage respiratory burst and cytokine production [90-91].

Other studies by the Forman lab using exogenous H$_2$O$_2$ showed that both the enhanced respiratory burst and cytokine production that were dependent upon it involved a transient elevation of intracellular free calcium that was released from annexin 6 when it dissociated from the inside of the plasma membrane [92]. Also it was shown that PC-PLC was activated by the elevated free calcium using either exogenous H$_2$O$_2$ [91] or silica [93]. Others have more recently suggested that PC-PLC release with high H$_2$O$_2$ concentrations was due to disruption of lipid rafts [94]. Thus, here whether silica would also disrupt lipid rafts and whether that was enhanced by the presence of iron was investigated.

The findings here show that H$_2$O$_2$ reacted with iron at the silica/macrophage interface to produce lipid peroxidation products. Lipid hydroperoxide production was blocked by MnP, strongly indicating H$_2$O$_2$ involvement, as MnP catalyzes the elimination of both O$_2^-$ and H$_2$O$_2$ (Figure 5A). Iron present on silicates predominately oxidize to Fe$^{3+}$ and can be reduced to Fe$^{2+}$ by O$_2^-$. This leads to the production of hydroxyl radical (OH), which due to its short life (it will react with a near diffusion limited rate with any molecule within one to two molecular radii), can contribute to the initiation of lipid peroxidation if it is produced in immediate proximity to a cell.
membrane (Figure 6). Others have implicated ‘OH in the initiation of cytokine production [95-96], but it is far too reactive to cross the cell membrane without causing lipid peroxidation. H$_2$O$_2$ as well as lipid peroxidation products produced in the membrane can diffuse into cells and act as second messengers in signaling in contrast [97]. Nonetheless, the level of production of H$_2$O$_2$ by the respiratory burst in the absence of silica is insufficient to initiate cytokine production. This was demonstrated by the failure of the ADP-stimulated respiratory burst, which stimulates similar O$_2^-$ production, as does silica, to induce cytokine production [98]. Nonetheless, other redox signaling pathways can be mediated by the H$_2$O$_2$ generated by the respiratory burst, and could activate the extracellular signal-regulated kinase and c-Jun N-terminal kinase, pathways [99-100]. Based on this difference, it was hypothesized that lipid peroxidation is responsible for initiating the signaling that results in production of cytokines in macrophages upon exposure to natural silica, which has not been studied previously.

Lipid peroxidation can cause reorganization of lipid rafts thereby affecting signaling [101]. Lipid rafts are large fraction sub-domains of plasma membrane that are rich in cholesterol and glycosphingolipids. Experimental evidence suggests that lipid rafts are involved in the regulation of signal transduction [102-103]; however, the involvement of lipid rafts in silica induced-proinflammatory mechanism has not been previously investigated. The results from immunocytochemistry (Figure 6) suggested that a disruption in the lipid raft organization in the macrophages upon treatment with a low dose of natural silica and iron-repleted silica was comparable with MβCD, which alters lipid rafts by removal of cholesterol [79]. In contrast, natural silica with DTPA added to chelate iron or use of iron-deleted silica did not disrupt the lipid rafts. Thus, it seems that iron plays a major role in disrupting lipid raft organization in silica treated macrophages.
Iron chelation with DFO and DTPA (Figures 5A, 7A, 7B) and scavenging of H\textsubscript{2}O\textsubscript{2} with MnP, which both inhibited lipid peroxidation and cytokine induction, also support the hypothesis that iron-mediated Fenton chemistry is the initiator of the signaling pathway resulting in TNF\textalpha{} and IL-1\textbeta{} induction. The Forman lab and others have shown that PC-PLC is an enzyme that is involved in the induction of cytokines in macrophages [58] and [67], which was also confirmed in the present chapter (Figure 7C). Previously, it was observed that exogenous addition of tertiary-butyl hydroperoxide [92] or silica [58] activated PC-PLC through an increase in intracellular free calcium concentration. The tertiary-butyl hydroperoxide-induced elevation of intracellular free calcium was through the dissociation of the phospholipid-binding protein annexin VI from the plasma membrane [92]. Subsequent studies have suggested that disruption of lipid rafts by lipid peroxidation caused annexin VI dissociation from lipid raft disruption [103]. Thus, the scheme in Figure 6 that summarizes the proposed mechanism for the pre-PC-PLC steps in the pathway from natural silica to initiation of cytokine production was investigated. The steps between the release of calcium from annexin VI and activation of PC-PLC are unknown. Others have studied the pathway from PC-PLC to cytokine activation, which is not detailed in the Figure 8. It involves the production of diacylglycerol by PC-PLC, activation by diacylglycerol of acid sphingomyelinase (ASM) to release ceramide, followed by sequential activation of ceramide activated protein kinase (CAPK), which activates NF-\textkappa{}B leading to cytokine transcription [67].

A novel pathway of iron-mediated lipid-raft disruption, leading to inflammatory cytokine production, was investigated in this work for silica exposed human macrophages at a realistic dose scenario. This work clarifies some of the ambiguities in the understanding of the silica-induced pro-inflammatory mechanism involving iron at a dose closer to real-life situation. The
key role of iron shown here suggests that one of the preventive measure to mitigate the respiratory effects caused by exposure to particles is to reduce the possibility of iron being on the surface of particles, such as engineered silica used in industry and to reduce exposure to iron coated particles, such as diesel exhaust.
2.6 Figures and figure legends

Figure 1: Particle characterization of natural (A) and iron-depleted silica (B). Primary particle diameter and size distribution (approximately 2 µm) were determined by SEM, surface area by
BET, and trace metal impurities by EDX and ICP-MS analysis. Particle size and shape were conserved upon acid etching, while iron was reduced from 40 ppb to undetectable levels.
Figure 2: Crystal structure analysis by powder XRD. Natural (A) and iron-depleted silica (B) matched the standard α-Quartz reference (C).
Figure 3: Determination of non-cytotoxic silica concentrations by MTT assay. THP-1 cell viability after exposure to different concentrations of natural silica particles was determined with MTT assay (*p<0.05). At 1 µg/ml, cell viability was close to the control (100%) cell viability value.
Figure 4: **Iron presence on natural silica particles markedly exacerbated NADPH oxidase dependent $O_2^-$ production.** (A) THP-1 cells were separately treated with (a) control (no silica), (b) natural silica (S) (1 μg/ml), (c) iron-depleted silica (S-Fe) (1 μg/ml), (d) 400 μM ADP, (e) 100 ng/ml PMA and (f) 100 ng/ml PMA and 30 μg/ml SOD; for 10 minutes to determine $O_2^-$ levels qualitatively. (B) Quantitatively, cells were also separately treated with iron-repleted silica (S+Fe) and also pretreated with 100 μM MnP (15 minutes), 20 μM DPI (30 minutes), and 1 mM DFO (1 hour), after which they were incubated with silica for 10 minutes. Treatment with S and S+Fe led to an increase in $O_2^-$, which was blocked upon treatment with MnP and DFO. The absorbance for control (no particles) was subtracted from the sample absorbance values (*p<0.05). (C) To further confirm the involvement of iron, iron chelator DTPA was used along with natural silica (S+DTPA), iron depleted silica (S-Fe+DTPA) and media (DTPA) and added to the cells. DTPA (1μM) was pretreated onto the cells for 30 minutes prior to the addition of
natural silica (S+DTPA), iron depleted silica (S-Fe+DTPA) and media (DTPA) separately. DTPA treatment did not affect S-Fe and media induced \( \text{O}_2^- \) production. The absorbance for control (no silica) was subtracted from the sample absorbance values.*Different \((p<0.05)\) from S, #different \((p<0.05)\) from S-Fe.
Figure 5: Iron presence on natural silica enhanced lipid hydroperoxides production. THP-1 cells were incubated independently with 1µg/ml of natural silica (S), iron-depleted silica (S-Fe) and iron-repleted silica (S+Fe), for 15 minutes. Pretreatment with 100 µM MnP (15 minutes), 1 mM DFO (1 hour) and 1µM DTPA (30 minutes) was done followed by silica addition for 15 minutes similar to NBT assay. DTPA was additionally also added along with S-Fe and media. Identical to superoxide production, treatment with S showed an increase in lipid peroxides.

*Different (p<0.05) from S, #different (p<0.05) from S-Fe.
<table>
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<th>Total %</th>
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<td>9</td>
<td>19</td>
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<tr>
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**Figure 6: Lipid raft organization disrupted in cells treated with natural silica particles with iron.**

Confocal images of the cells pre-labeled with fluorophore-conjugated CTX-B (green) following simulation of cells for 15 seconds with different silica treatments with or without iron and iron chelator DTPA and (5mM) MβCD (pretreated 30 minutes separately before the addition of silica). Nuclei were stained with DAPI. The table represents images with 50 cells randomly counted from the entire coverslip plated with cells. Increased lipid raft disruption similar to that of the positive control, MβCD, can be observed in the cells treated with S and S+Fe (iron present) as compared to S-Fe, S+DTPA, S-Fe+DTPA (iron absent) where cells showed a more concentrated distribution of GM1-associated domains.
**7A**

![Graph showing IL-1β mRNA levels across different treatments.](image)

**7B**

![Graph showing TNFα mRNA levels across different treatments.](image)
mRNA (fold control)

Cytokines

- IL-1β
- TNFα

Silica
Silica + D609
Silica + MnP

* indicates significant difference
Figure 7: Silica increased cytokine activation is PC-PLC dependent.
(A, B) THP-1 cells were treated independently with 1 μg/ml of natural (S), iron-depleted silica (S-Fe) and iron-repleted silica (S+Fe). Separately cells were also pretreated with 1 mM DFO (1 hour), 1 μM DTPA (30 minutes) before the addition of silica. The relative mRNA levels of IL-1β and TNF α were determined with RT-PCR. Similar response was observed even after 6 and 12 hour treatment (data not shown). Similar to O$_2^-$ and lipid peroxidation, both the cytokines amplified significantly in the presence of S, S+Fe in contrast to S-Fe. Addition of chelator DTPA decreased this effect. *Different (p<0.05) from S, #different (p<0.05) from S-Fe. (C) PC-PLC dependence was determined by pre-treating the cells with 40 μM D609 followed by addition of 1 μg/ml of natural silica for 6 hours. Additionally cells were also pretreated with 100 μM MnP for 1 hour before silica treatment. Both MnP and D609 decreased the silica initiated cytokine production significantly (*p<0.05). (D) Silica particle surface/cell contact is required for cytokine production. Confocal images of THP-1 macrophages immunostained for IL-1β and TNF-α were observed. The differentiated cells were fixed and stained with rabbit polyclonal anti IL-1β and TNFα (1:100) as primary antibody. Alexa Fluor 568 donkey anti - rabbit IgG was employed as secondary antibody. Nuclei were stained with DAPI. White arrows indicate the presence of silica particles on the surface of the macrophage membrane during cytokine production.
Figure 8: (1) Silica binds to the macrophage receptor resulting in signaling for activation of NADPH oxidase complex, which (2) produces $O_2^-$. (3) $O_2^-$ dismutates in the acidic environment of the phagosome to $H_2O_2$ and oxygen. (4) Transition metals, such as $Fe^{3+}$, on the silica particles can be reduced by superoxide. (5) $H_2O_2$ reacts with $Fe^{2+}$ via Fenton chemistry to produce hydroxyl radical (OH). (6) The ‘OH or an iron-oxygen complex can initiate lipid peroxidation when in close proximity to the cell membrane.
CHAPTER 3

The effect of particle size, phase and iron on low dose engineered silica-induced pro-inflammatory production

3.1 Abstract

Engineered silica nanoparticles, due to their unique chemical and physical properties at the nanoscale, can be exploited to produce functionalized materials with varied applications. However, increasing evidence suggests that these properties can also contribute to the production of reactive oxygen species as observed with natural silica. Currently, the pro-inflammatory mechanism of exposure to realistic low doses of engineered silica are not well understood and need further elucidation. Furthermore, since engineered nanoparticles are uniform in size, shape, and surface chemistry, they offer the opportunity for isolating and investigating the effect of particle properties such as iron, particle size, and phase on silica-induced inflammation.

Here, the influence of particle size, surface iron, and phase on engineered silica-induced inflammatory responses under a non-cytotoxic dose (similar to that used in the previous chapter showing natural silica-induced pro-inflammatory mechanism), using human-derived THP-1 macrophages, was investigated. Nano- (50 nm) and micro-sized (2 μm) engineered silica particles were iron doped and characterized for size, morphology, surface iron, phase, and aggregation. The results indicated that iron-doped 50 nm particles induced elevated levels of superoxide, lipid peroxidation, and cytokine (IL-1β and TNFα) production in the macrophage membrane compared to 50 nm undoped particles, as well as iron-doped and undoped 2 μm particles at 1 μg/ml, confirming the importance of particle size and iron. The use of iron chelator, Diethylene Triamine Pentaacetic Acid (DTPA), decreased cytokine production, validating the role of iron. Furthermore, to determine whether engineered silica stimulated the
cytokine production through Phosphatidylcholine-specific Phospholipase C (PC-PLC), Tricychodecan-9-yl-Xanthate, a competitive inhibitor of PC-PLC, was used. This inhibited engineered silica-stimulated cytokines production similar to natural silica. Importance of phase was determined by comparison of the 2 μm crystalline natural silica with 2 μm engineered amorphous silica particles that increased superoxide, lipid peroxidation, and cytokine production upon exposure to crystalline silica. This study suggests that at 1 μg/ml dose, size, iron, and phase affected silica-induced inflammatory production via the activation of PC-PLC through macrophage lipid membrane damage similar to natural silica.

3.2 Background and related work

The rapid development of engineered/manufactured nanophases, such as silica, titania, and carbon nanotubes has added a new potential source of environmental and workplace airborne toxicity [104]. Unlike ambient particulates (diesel exhaust, silicates, silica), these are generally homogeneous in size, shape, and phase. Silica, in particular, has desirable material properties such as thermal and chemical stability and has been widely used in the form of nanoparticles for various applications, such as drug delivery, cancer therapies, biosensors, gene delivery, biomedical imaging and as catalyst supports [105-108]. Transition metals, such as iron, may also be added to engineered silica particles to make them more beneficial for bio-medical and industrial applications, but the presence of metals in even low concentrations can also contribute to their toxic nature as shown by studies conducted for silica [109] and other materials [110].

Apart from metal-dependent toxicity studies, previous studies have also demonstrated a size-dependent toxicity. Chen et al. [111] observed that both nano- and micro-sized silica caused pulmonary inflammation in rats. Lin et al., Akthar et al. and Choi et al. [112] [113-114] demonstrated oxidative stress and depletion of glutathione levels in A549 and BEAS-2B cells
upon exposure to amorphous silica nanoparticles in a dose- and time-dependent manner. Both Napireska et al. [115] and Kyung Yu et al. [116] showed a size-dependent cytotoxicity of silica nanoparticles with surface area and size being important determinants of toxicity. Wottrich et al., [117] with A549 cells, reported toxicity of synthesized 60 nm silica nanoparticles, but only at high concentrations of 200 µg/ml that can easily lead to macrophage overload.

The majority of studies stated above and others have however, mainly focused on silica nanoparticle induced-cytotoxicity and have not addressed the mechanistic features of the inflammation, especially the upstream pathways through which particle parameters along with metal contaminants influence engineered silica’s pro-oxidative and pro-inflammatory signaling in macrophages at low non-cytotoxic doses. This is a key area to explore, since engineered silica particles are being used in various applications from therapeutics to the semiconductor industry, and it is important to understand the chronic short- and long-term effects of exposure to these particles for safe handling and to mitigate risk. In the work investigated in the previous chapter, natural ambient silica particles were shown to produce superoxide (O$_2^-$), and hydrogen peroxide (H$_2$O$_2$). In the presence of metal ions such as Fe$^{2+}$, H$_2$O$_2$ can react with it to produce the hydroxyl radical, which can damage the lipid membrane and DNA [110, 118-119]. Further, it was demonstrated that phosphatidylcholine-specific phospholipase C (PC-PLC) may be involved in natural, crystalline silica-stimulated induction of TNF-α and IL-1β inflammatory molecules [58]. However, the extent of inflammatory production by engineered amorphous particles could be lower than natural crystalline silica particles, due to silica surface dehydroxylation, as shown by other studies [120].

In this context, the aims of this chapter were to a) determine the contribution of particle size, phase and metals, in particular iron, to the pro-inflammatory nature of engineered
amorphous, silica particles, b) obtain a better understanding of the pro-inflammatory signaling induced in the macrophages by exposure to well-controlled particles at a non-cytotoxic concentration, and c) examine whether the inflammatory pathways observed in cells with natural silica particles are similar to those of engineered silica particles.

3.3 Materials and methods

3.3.1 Reagents and materials

Engineered silica nano- and micro-sized particles used in this study were spherical amorphous silica particles with an average primary particle size of 50 nm and 2 µm, respectively, purchased from Microspheres-Nanospheres (Cold Spring, New York, USA). Materials used were Diphenyl-1-pyrenylphosphine (DPPP) (Invitrogen), Tricychodecan-9-yl-xanthate (D609) (BIOMOL International), TRIZOL reagent (Life Technologies), TaqMan reverse transcription reagent and SYBR Green PCR Master Mix (Applied Biosystems). Manganese (III) tetrakis (N-ethylpyridinium-2-yl) porphyrin (MnP) [1] was kindly provided by Dr. James Carpo, National Jewish Health (Denver, USA). All other chemicals, unless specified were purchased from Sigma (analytical grade).

3.3.2 Silica particles and characterization

According to the manufacturer's specifications, the particle solutions contained spherical silica micro and nano-particles with an average primary particle size of 2 µm and 50 nm, respectively. Prior to the exposure, the particle suspensions were cleaned of synthesis residues, solvents, and lipopolysaccharide contamination by autoclaving the suspensions. Particle suspensions were then concentrated and dried for 24 hours. The final silica material was weighed and stored. The uniformity of the particle sizes, size distribution, and morphologies were measured using a JEOL
2010 High-Resolution Transmission Electron Microscopy (HRTEM) equipped with LaB₆ filament (200kV and 120kV), Scanning Electron Microscope (SEM) (FEI Quanta 200 SEM) equipped with a tungsten filament, and Energy-dispersive X-ray spectroscopy (EDX) detector with ultrathin window and 132 eV energy-resolution. X-ray powder diffraction (XRD) patterns were collected on a PRO PANalytical X-Ray Diffractometer to confirm the amorphous phase of the 2 µm silica particles. A selected area diffraction pattern was collected on the HRTEM to confirm the amorphous phase of the 50 nm nanoparticles. Iron adsorption onto the silica was determined by using an Agilent 7500 Inductively Coupled Plasma Mass Spectrometer (ICP-MS) (detection limit for iron: 1.5 µg/L or ppb), along with a colorimetric measurement as described by Ghio et al. [2]. The surface area of the particles was determined by the Brunauer-Emmett-Teller (BET) method. Both 2 µm and 50 nm suspensions were prepared in phosphate buffered saline (1X PBS) and dispersed by a sonicator to avoid aggregation. In each study, the suspension was freshly prepared, diluted, and then immediately treated to the THP-1 cells.

3.3.3 Iron doping protocol
To determine the amount of Fe³⁺ doped on the surface of 2 µm and 50 nm silica particles, these particles were exposed to FeCl₃ dissolved in degassed water. 1.0 ml of 1 mM FeCl₃ solution was added to 1.0 ml of silica (2 mg silica/ml). Suspensions were vortexed, agitated for 24 hours, and centrifuged at 1,200 g for 10 minutes. To avoid the formation of oxyhydroxides, water used for dilutions was degassed in argon gas for 24 hours and glassware was washed with 1% HNO₃. The supernatant was then assayed for iron by the method described by Ghio et al. [59]. To quantify the surface iron absorption, standards and samples (0.8 ml) were acidified with 0.1 ml of 25% (wt/vol) trichloroacetic acid; 0.1 ml of 2.5 M potassium thiocyanate was added, and the absorbance was determined at 480 nm. The amount of iron adsorbed was calculated as the
difference between the initial and final concentrations in the solution. Engineered silica particles exposed to 1 mM FeCl₃ were washed with distilled water and dried. Measurements were done in triplicate.

3.3.4 Cell culture

THP-1 cells (Human acute monocyctic leukemia cell line) purchased from American Type Culture Collection, TIB-202 were cultured in T25 flasks (Fisher Scientific) with RPMI 1640 culture medium supplemented with 10% Fetal Bovine Serum (FBS) (Omega Scientific), β-mercaptoethanol, 100 μg/ml penicillin, and 100 μg/ml streptomycin at 37°C in a humidified 5% CO₂ atmosphere. In recent years, these cells have been widely established as an in vitro model for native monocyte-derived macrophages in studies of inflammatory disease by particle inhalation [121-122]. Prior to the experiments, the monocytes were transferred to cell-culture plates, coated with 5% bovine serum albumin in phosphate saline buffer, dried and gently rinsed twice with water into the desired concentration depending on the experiment and differentiated into adherent macrophages by treatment with 50 ng/ml Phorbol 12-Myristate-13-Acetate (PMA) for 3 days.

3.3.5 Determination of non-cytotoxic exposure- 3-(4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazoliumbromide (MTT) assay

THP-1 cells were differentiated 3×10⁵ cells/ml per well in 12-well tissue culture plates. After 22 hours of particle exposure, % viability was quantified by incubating the cells for 2 hours at 37°C with MTT (5 mg/ml). The resulting colored product was solubilized in MTT solubilization solution (10% Triton-X 100 and 0.1N HCl in acidic isopropanol). The absorbance was measured at 550 nm and the background absorbance was measured at 690 nm using a microplate reader.
(Molecular Device Corp., CA). The non-cytotoxic concentration of 1 µg/ml was determined by this assay and was used in all the experiments.

3.3.6 Modified colorimetric nitroblue tetrazolium (NBT) Assay

To determine the superoxide production, colorimetric NBT assay was performed as described by Rook et al. [73]. THP-1 monocytes (3×10⁵/well) were differentiated into the macrophages in a 12-well culture plate. After pretreatment with 2.5 mM NBT solution in warm Krebs Ringer Phosphate Buffer (KRP) for 30 minutes, exposure to 0.05, 0.1, and 1 µg/ml of 2 µm and 50 nm undoped and iron doped silica particles was carried out. 50 ng/ml PMA was used as a positive control. Cells were washed with warm PBS to remove the extracellular NBT and then air-dried. The amount of NBT deposited inside the cells was then quantified by adding 120 µL 2 M Potassium Hydroxide (KOH) and 140 µL Dimethyl Sulfoxide (DMSO) to dissolve the cell membrane. The resulting blue formazan was mixed and transferred to a 96-well plate to read the absorbance at 360 nm.

3.3.7 Diphenyl-1-pyrenylphosphine (DPPP) fluorescence

THP-1 (100,000 cells/well) monocytes were differentiated into macrophages in a 96-well culture plate. Cells were incubated with 5 µM DPPP dissolved in 0.05% DMSO for 15 minutes in the dark and washed twice with 1X PBS buffer to completely remove extracellular DPPP. After an exposure of 1 µg/ml of 2 µm and 50 nm undoped and iron doped silica particles to the cells for 15 minutes, the increased emission fluorescence intensity of DPPP oxide (DPPP= O), was measured at an excitation of 351 nm and an emission of 380 nm. As a positive control, 100 µM cumene hydroperoxide was added.
3.3.8 Real-time polymerase chain reaction (RT-PCR) assay

Quantification of cytokines TNF-α and IL-1β mRNA was determined on a Cepheid 1.2 RT-PCR machine using the following primers: Glyceraldehyde 3-phosphate dehydrogenase (GAPDH), sense 5’-ACCCCAATGTATCCGTGT-3’ and antisense 5’-TACTCCTTGGAGGCCATGT-3’, TNF-α, sense 5’- GCCTCTGTGCTTCTTTTGTA-3’ and antisense 5’-GCAACCTTATTTCTCGCCA-3’ and IL-1β, sense 5’-CGACACATGGGATAACGAGGCTT-3’ and antisense 5’ TCTTTCAACACGCAGGACAGGTA-3’ along with 5 µl of reverse transcription reaction product and 12.5 µl SYBR Green PCR Master Mix. After 3 hours, 6 hours, and 12 hours the RNA was extracted from THP-1 differentiated macrophages (2 x 10⁶/well) treated with 1 µg/ml of the investigated particles according to the manufacturer’s protocols.

3.3.9 Statistical analysis

All data were expressed as the mean ± standard deviation. One-way ANOVA and one-tailed unpaired student's t-test were used for significance testing, using *p < 0.05. All the experiments were conducted with replicates, so the data are the means of three experiments, each using three experimental points, with control values subtracted from that of the sample, when compared to undoped silica.

3.4 Results

Inflammatory response induced by particles is mostly dictated by size, surface area, morphology, phase, charge, and chemical composition [123]. The objectives of this chapter were to investigate the relationship between important particle parameters - size, phase, surface iron, and inflammatory mediators - that alter the cell membrane integrity at a low non-cytotoxic dose using
well-controlled silica particles, and to also investigate the link between oxidative stress and cytokine production induced by nano- and micro-sized engineered silica.

3.4.1 Particle characterization

The physical properties of the micro- and nano-engineered silica particles were summarized in Table 1, Figure 1A, 1B and 2A. The mean size and size distribution of the “as received/undoped” particles were observed by Scanning Electron Microscopy (SEM) and Transmission Electron Microscopy (TEM) by measuring the diameter of about 400 particles in the micrographs (Table 1, Figure 1B a,b). This was consistent with the measurements provided by the suppliers, within reported variances. The surface area for 2 μm and 50 nm particles was evaluated by Brunauer-Emmett-Teller (BET) method (Table 1). Dynamic Light Scattering (DLS) measurements conducted for the 50 nm particles in the presence or absence of RPMI 1640 media after sonication indicated that their hydrodynamic diameters weren't larger than the size range estimates measured by TEM (Table 1).

A study by Brunner et al. [124] discussed and showed the need for using reliable methods to observe particle dispersions as it can cause discrepancies in data interpretation. In the present study, particles were characterized before and after dispersion to the cell culture media just prior to cell exposure. Due to the shorter exposure times used in this study in the cell culture media and sonication prior to it, no dispersing agents were used as no aggregate formation was observed. Uniform dispersal of particles allowed for control over determining size-dependent effects (Table 1, Figure 1Ab, 2Ab). The phase of the 2 μm and 50 nm particles was amorphous, as clearly shown from the X-ray Diffraction (XRD) broadened peaks and diffuse or halo electron diffraction pattern [125] (Figure 1Ac, 2Ac). The use of characterized silica particles with
uniformity in the average size and distribution provided a controlled platform for data comparison.

Batches of 2 μm and 50 nm particles were doped with iron and comparisons were made with their respective undoped silica particles. The concentration of the iron, doped on the surface of the silica particles, was measured by both calorimetric assay and Inductively Coupled Plasma Mass spectroscopy (ICP-MS). Iron was mainly chosen as the metal dopant to silica as it is one of the most common and important metals added to nanomaterials (for example, for catalysis) and is capable of redox cycling [126], and iron impurities are also present in the raw materials or precursors used for particle synthesis. Procedures for iron-oxide coating of silica particles are well established and generally involve precipitation of the Fe\(^{3+}\)-oxide phase from aqueous solution. The calorimetric measurements for iron concentrations were calculated by the method described by Ghio et al., [59] for 2 μm and 50 nm silica were 34.009 μM and 54.027 μM of iron per gram of silica. The ICP-MS iron concentrations for 2 μm (36.75 μM/g silica) and 50 nm (52.34 μM/g silica) doped particles were comparable to the calorimetric data. As a function of surface area, the iron concentration was more for the 2 μm particle (0.027 moles/m\(^2\) of silica versus 50 nm particles (0.009 moles/m\(^2\)), however, moles of iron were normalized. Both 2 μm and 50 nm silica particles contained significant amounts of complexed iron on the surface from the solutions of FeCl\(_3\), although 50 nm particles complexed more Fe\(^{3+}\) than the 2 μm particles due to their larger surface area to volume ratio. In comparison, based on the manufacturer's data and ICP-MS analysis, iron on the undoped 2 μm and 50 nm particles was below detectable limits. Also, no iron was detected on the undoped 2 μm and 50 nm particles when measured by Energy-dispersive X-ray spectroscopy (EDX) analysis (Figure 1Aa, 2Aa).
3.4.2 Effect of size and phase on engineered silica in O$_2^-$ and lipid peroxidation production

In the first set of experiments, both 2 μm and 50 nm particles, along with their respective iron doped particles, were investigated for their stimulation of O$_2^-$, and thus hydrogen peroxide (H$_2$O$_2$) production, in the cultures of human monocytes-derived macrophages in vitro. NBT formazan absorbance, indicative of O$_2^-$ production in the cells, doubled significantly in macrophages after a 10 minute exposure to undoped 50 nm silica particles, when compared to undoped 2 μm particles of the same mass concentration of 1 μg/ml (Figure 3). The O$_2^-$ production was 2- and 3-fold higher in macrophages exposed to 2 μm and 50 nm particles than in the control cells (no particles). At extremely non-cytotoxic doses of 0.05 and 0.1 μg/ml, the difference was not significant. Levels of O$_2^-$ observed were comparable to 50 ng/ml PMA, a known stimulus of the respiratory burst [76]. No comparison of size-dependent effects of O$_2^-$ production from the iron-doped batches of the micro- and nano-sized particles was performed due to the difference in their iron concentrations. When expressed on a mass concentration (μg/ml) basis, the dose-response relationship for O$_2^-$ production supported a size-dependent effect, with increased production as particle diameter decreases (Figure 3). Similar to O$_2^-$ production results, lipid peroxide production showed a size-dependent effect. Lipid peroxidation after 15 minutes of exposure to a low non-cytotoxic dose of 1 μg/ml was significantly increased for 50 nm undoped particles over the same mass concentration of 2 μm particles (Figure 3). As a positive control, cumene hydroperoxide (CHO) (100 μM) was used as demonstrated in previous studies [77]. To establish the relationship between O$_2^-$ and lipid peroxidation production, the cells were pretreated with 100 μM Manganese (III) tetrakis (N-ethylpyridinium-2-yl) porphyrin (MnP). MnP is a superoxide dismutase (SOD)/catalase mimic that scavenges H$_2$O$_2$ [127]. This reduced the lipid peroxidation significantly in the macrophages. At the same non-cytotoxic dose,
when the 2 μm crystalline natural silica particles assessed in chapter 2 were compared to the similar sized 2 μm engineered amorphous silica, all the cellular responses observed for particle size dependency were significantly lower for the amorphous silica particles. This suggests a phase dependency for the silica-induced pro-inflammatory mechanism, as well.

3.4.3 Effect of surface iron on engineered silica in O$_2^-$ and lipid peroxidation production

Previous investigators such as Limbach et al. [14] have shown that iron-containing nanoparticles promoted reactive oxygen species production within human lung epithelial cells. In this study with human derived macrophages, even the presence of low levels of iron on the 2 μm and 50 nm particles, 34.009 μM/g and 54.027 μM/g of iron/g respectively, increased O$_2^-$ production significantly in THP-1 cells, compared to the cells without any particles and cells with undoped particles (Figure 3). The difference in the iron loading onto the surfaces of nano- and micro-sized particles as seen in Table 1 could be mainly because of the increase in surface area as particle size is decreased [128]. This variance however, did not affect the current study as the main focus was to investigate the size-dependent effects of as received/undoped engineered silica, and to understand the influence of metals on both engineered nano- and micro-sized silica’s pro-inflammatory effects individually. Similar to size dependency, the presence of iron on the micro- and nanoparticles leads to a higher O$_2^-$ production by cells in contrast to their counterparts. To investigate whether the presence of iron or change in particle diameter also caused lipid peroxidation, lipid peroxidation damage was quantified by using DPPP, which is specific to lipid hydroperoxides, but does not react with hydrogen peroxide [74]. Iron on the 2 μm and 50 nm particles caused a 2- and 3- fold increase in lipid peroxidation over the control, as compared to undoped 2 μm and 50 nm particles that caused a 1.3 and 1.7 fold increase over the control (Figure 4).
3.4.4 PC-PLC dependent iron mediated-engineered silica-induced inflammatory mediator production

To investigate the mechanism of particle-induced cytokine production, relative mRNA expression levels of suspect genes, the two critical cytokines involved in silica-associated inflammation were measured by RT-PCR. The levels of mRNA expressions were higher in THP-1 cells within 3 hours of exposure to 1 μg/ml of 2 μm and 50 nm silica particles, but the expression levels did not increase at further time points of 6 and 12 hours (data not shown). Similar to O₂⁻ and lipid peroxidation, in the presence of doped iron and a smaller particle size (50 nm), both of the inflammatory mediators elevated strikingly as shown in Figure 5. Addition of the iron chelator DTPA, along with engineered doped 2 μm and 50 nm silica, significantly lowered the levels of both cytokines (Figure 5). Lipopolysaccharide (LPS) was used as a positive control as it is a known inducer of lipid peroxidation. Prior to silica (1 μg/ml) exposure, pretreatment with MnP (100 μM) decreased TNF-α and IL-1β production in the macrophages, showing the dependence of cytokine induction and lipid peroxidation on O₂⁻ generation. To determine the involvement of PC-PLC, which was previously showed to be associated with natural silica-induced cytokine production in chapter 2, the macrophages were pretreated with 40 μM D609 (Tricyclodecan-9-yl-Xanthogenate), a PC-PLC inhibitor, for 10 minutes prior to exposure to 1 μg/ml of iron doped 50 nm and 2 μm engineered silica particles. This inhibited engineered silica-induced IL-1β and TNF-α transcription in THP-1 cells (Figure 5 A,B,C,D), indicating PC-PLC involvement.

3.5 Discussion and conclusion

In the past decade there has been an increase in the number of studies reporting nanoparticle toxicity and oxidative stress [11, 14, 112, 129]. However, to date very few studies have
addressed the cellular consequences of oxidative stress leading to inflammation at non-cytotoxic doses. Using a high toxic dose could overwhelm macrophages and impair their phagocytic function and gene expression [130-132]. The results with engineered micro- and nano-sized silica particles showed that at a low non-cytotoxic dose of 1 µg/ml; O$_2^-$, lipid peroxide and cytokine production increased in macrophages in a size- and iron-dependent manner.

To determine the upstream micro- and nano-sized engineered silica-macrophage interaction mechanism, the peroxidation of lipid membrane, activation of inflammatory mediators (IL-1β and TNF-α), and role of PC-PLC enzyme were investigated and comparisons drawn with natural silica particle-induced inflammatory response [118]. A non-cytotoxic dose of 1 µg/ml of silica, determined from MTT reduction assay results (Figure 2B), consistent with earlier reports for onset of cyto-toxicity of engineered silica particles between the range of 10-100 µg/ml [114, 116], was used in order to determine the inflammatory effects of both 2 µm and 50 nm silica particles. This dose again was also closer to that of the natural silica dose that was studied in chapter 2 and also relatively closer to the realistic exposure levels of natural silica in PM, based on the calculations performed in section 1.2.4.

Exposure to 50 nm undoped particles significantly increased O$_2^-$, lipid peroxide and cytokine production over 2 µm undoped particles, which is expected due to the larger surface area per unit mass of the 50 nm particles, indicating an influence of particle size (Figures 3,4,5). Previous toxicological studies have indicated a possible relation between particle size and cytotoxicity [133-134]. This study shows that even at a low non-cytotoxic mass dose of 1 µg/ml there is evidence of particle size influence over oxidative stress, lipid peroxidation and inflammation. The evidence of particle size dependency was prominent, as even with the lower surface iron concentration for 50 nm doped particles, these particles resulted in higher
productions compared to the 2 µm particles (Figures 3,4,5). The work in chapter 2 and others have observed that the presence of iron in natural silica particles exacerbates reactive oxygen species production [39, 118, 135]. The findings in Figure 3 confirm that engineered silica particles follow a similar behavior to natural silica particles with respect to the influence of iron on O$_2^-$ production. The iron doped micro- and nano-sized silica particles also showed a statistically significant O$_2^-$, lipid peroxide and cytokine (IL-1β and TNFα) induction, over undoped silica particles, their counterparts (Figures 4 and 5).

The role of iron in increasing O$_2^-$, lipid peroxidation, and cytokine production was also confirmed when the iron chelator DTPA reduced pro-inflammatory cytokines in the macrophages (Figure 5). This data suggests that iron present on the silica particle exacerbates the O$_2^-$, and thus H$_2$O$_2$ production in the macrophages. Further membrane damage observed in the macrophages could be due to the production of hydroxyl radical (HO·) through Fenton reaction (Fe$^{2+}$+H$_2$O$_2$ → Fe$^{3+}$+OH$^-$+HO·) as shown in the previous chapter. To further clarify the possible upstream oxidative signaling pathway, the cultured macrophages were pretreated with a free radical scavenger, MnP. Interestingly, pretreatment with MnP decreased the subsequent lipid peroxide and inflammatory cytokine production in the macrophages-induced with both 2 µm and 50 nm silica particles (Figure 4). This was similar to the previous observation with natural silica. Further studies were conducted to elucidate the mechanism between lipid peroxidation and pro-inflammatory mediator production. It was established in the work in the previous chapter with natural silica that PC-PLC enzyme is required for the induction of cytokines [58, 118]. Others have shown that PC-PLC acts through Nuclear Factor-kappa B (NF-κB) signaling pathways, which are known to be involved in the upregulation of key inflammatory factors [136-137]. D609, a PC-PLC inhibitor was used to determine if PC-PLC
played a role in the engineered silica particle-cytokine activation [93, 138]. This inhibited both micro- and nano-sized silica-induced IL-1β and TNFα transcription (Figure 5). Further work is, however, warranted to understand the PC-PLC activation from lipid peroxidation products. Involvement of lipid rafts disruption is a possible source [118].

The role of material phase was confirmed by comparing superoxide, lipid peroxide, and cytokine production of natural crystalline silica; studied in chapter 2; with the engineered amorphous silica studied in this chapter. As observed by other studies with silica and other types of materials [120, 139], the results here also showed increased production of the various responses measured with 2 μm crystalline silica compared to 2 μm amorphous silica. Crystalline particles are characterized by a few very reactive sites, absent on the amorphous specimens, thus, leading to higher oxidant capacity in the similar size range [45]. However, a similar effect was not observed when comparing 50 nm amorphous silica particles with 2 μm crystalline particles due to the size interference. Since the dose that was delivered was on a mass basis, the number of particles per unit mass was much more for 50 nm particles. A more effective dose basis to investigate this setting would be based on surface area.

The present investigation has demonstrated that engineered silica particles at low non-cytotoxic doses exhibit a strong particle size, phase, and iron-dependent lipid peroxidation-mediated inflammatory response in THP-1 human macrophages. Particle size, along with iron, aggravated pro-inflammatory mediator production even at a low non-cytotoxic dose of 1 μg/ml. It was further demonstrated that similar to natural silica, PC-PLC enzyme could be involved in engineered silica-induced cytokine (TNF-α, IL-1β) production.

This study helps understand some key particle parameters triggering the underlying pro-inflammatory mechanism that are largely unknown for engineered particles at a dose limit that
would not lead to complete cell death and alteration of gene expression levels. Elucidation of this pathway will provide useful information for hazard assessment for people and workers coming in contact with engineered silica for day-to-day and occupational purposes. It will also provide additional analysis from a more controlled (with respect to composition and size) materials system for assessing the effect of surface transition metals on particle-induced inflammation.
3.6 Figures and figure legends

Table 1: Engineered silica 2 µm and 50 nm nanoparticle characteristics. Sizing data were obtained using SEM, TEM, and DLS. Iron concentration and surface area were measured by ICP-MS and BET analysis.
Figure 1A: Engineered 2 μm silica characterization studies are described here. (a, b) EDX pattern and SEM micrograph of the sample, c) XRD pattern of 2 μm of engineered silica. Particle size and morphology were found to be uniform, no measurable iron was observed, and the phase was amorphous in nature.
Figure 1B: Particle characterization of size distribution and aggregation. (a, b) 2 μm and 50 nm silica particle size distributions as determined by analysisPRO software using SEM micrographs, (c, d) Optical micrographs of 2 μm engineered silica in PBS and cell culture media. Particles were well dispersed in the buffer, but few aggregates were observed in the media.
Figure 2A: Engineered 50 nm Silica characterization studies are described here. (a,b,c) EDX pattern, TEM micrograph and selected area diffraction of 50 nm engineered silica. Particle size and morphology were found to be uniform, while iron contaminants were below the detection limit and the phase was found to be amorphous in nature.
Figure 2B: Determination of non-cytotoxic silica concentrations. MTT assay was performed to determine the non-cytotoxic exposure levels of engineered 2 µm and 50 nm silica particles. The cell viability was determined as a percentage control. Values are mean ± SD from three independent experiments. Significance indicated by *p < 0.05 when compared to control (no particles).
**Figure 3:** Iron presence and reduction in particle size increases $O_2^-$ production. Effect of size and surface iron of engineered silica A) 2 µm, B) 50 nm particle induced THP-1 macrophage $O_2^-$ production after 10 minutes at low non-cytotoxic concentrations. The absorbance for control (no particles) was subtracted from the sample absorbance values. Values are the mean ± SD from three independent experiments. *p < 0.05 when compared to undoped silica.
Figure 4: Iron presence and reduction in particle size increases lipid peroxidation production.

Effect of size and surface iron on engineered silica A),B) 2 μm and 50 nm induced- THP-1 lipid peroxidation production in the macrophages. To determine the involvement of H₂O₂ in the lipid peroxidation production, macrophages were also separately pretreated with 100 μM MnP, a SOD/catalase mimic, for 15 minutes, prior to silica treatment (10 minutes). The absorbance for
control (no particles) was subtracted from the sample absorbance values. Values are the mean ± SD from three independent experiments. Significance indicated by *p < 0.05 when compared to undoped silica.
Figure 5: Iron presence and reduction in particle size increases cytokine production via PC-PLC. PC-PLC dependent iron mediated-engineered silica-induced inflammatory mediator
production after 3 hours. A similar response was observed even after 6 and 12 hour treatments (data not shown). A and B represent IL-1 β and TNF-α response induced in the macrophages by exposure to engineered 2 µm silica. C and D represent IL-1 β and TNF-α response induced in the macrophages by exposure to engineered 50 nm silica. Values are the mean ± SD from three independent experiments (*p < 0.05). Separately cells were also pretreated with 1 µM DTPA (30 minutes) before the addition of silica. The relative mRNA levels of IL-1β and TNF α were determined with RT-PCR. Similar to O2− and lipid peroxidation, both the cytokines amplified significantly in the presence of iron. Addition of chelator DTPA decreased this effect. PC-PLC dependence was determined by pre-treating the cells with 40 µM D609 followed by addition of 1 µg/ml of natural silica for 6 hours. Additionally cells were also pretreated with 100 µM MnP for 1 hour before silica treatment. Both MnP and D609 decreased the silica initiated cytokine production significantly (*p<0.05).
CHAPTER 4

Microscopic evidence of human macrophages interacting with micro- and nano-silica particles at a high and low dose

4.1 Abstract

The objectives in this study were to microscopically observe the effect of particle size on macrophage uptake of engineered micro- and nano-amorphous silica particles and the effect of a cytotoxic and a non-cytotoxic dose on the macrophages. Observations with optical and Transmission Electron Microscopes (TEM) indicated that THP-1 derived macrophages at 24 hours interacted with and phagocytosed 2 µm and 50 nm silica particles. The 2 µm silica particles, as viewed by optical microscope, accumulated in the intracellular phagosomes and cytoplasm, but the bulk of them were observed to be surrounding the cell membrane periphery. The majority of the 50 nm particles, however, as observed by TEM, were actively accumulated into intracellular phagosomes and phagolysosomes. Although, some of the nanoparticles were found isolated in the cell cytosol without being bound by the phagosome membrane, suggesting some degree of inconsistency in the phagocytosis mechanism. This suggested that both the micro- and nano-engineered silica particles were phagocytosed by the macrophages, although some particles seemed to bypass this clearance mechanism. The internalization was, however, observed to be greater for 50 nm particles and therefore size-dependent.

Secondly, the effect of high and low particle dose was also observed microscopically to understand whether high particle dose, apart from inflammatory response also affects particle internalization mechanism. At a high cytotoxic dose of 100 µg/ml, due to an excess of phagocytosable particles, macrophage overburden was observed with increases in macrophage lysosomes (containing degrading enzymes to digest the particles), suggesting an heightened
clearance mechanism resulting in the possibility of excessive inflammatory response as a result of macrophage cell-death and not nanoparticle-triggered inflammatory response. The results suggested that amorphous silica particles were mainly phagocytosed by macrophages regardless of the particle size, but with a size-dependent internalization. Furthermore, high cytotoxic-doses could alter macrophage clearance and thus, possibly downstream cellular mechanisms as well.

4.2 Background and related work

As stated in chapter 3, engineered micro- and nano-sized silica particles have various applications. Silica nanoparticles are produced on a large industrial scale for various purposes, such as cosmetics, food additives, varnishes, and drugs. The ability of inhaled engineered silica particles to initiate lung toxicity and inflammation is still not well known, and continues to receive attention, especially since inflammation is a critical aspect for the development of lung disease. This dissertational study showed a novel pathway whereby lipid membrane damage, especially lipid raft disruption, could be responsible for the initiation of silica-induced inflammation (chapters 2 and 3), with particle size, phase, and iron playing a crucial role at a low non-cytotoxic dose.

The primary aim of this investigation was to study the effect of particle size of engineered silica particles on their internalization by macrophages at a low non-cytotoxic and a high cytotoxic dose. Particle size is considered to be an important parameter in designing drug delivery systems as it can alter the cellular uptake, toxicity, and inflammatory mechanisms induced by the particles. Previous studies have reported the cellular uptake of engineered silica nanoparticles leading to their accumulation in the nucleus and cytoplasm of non-phagocytic cells, such as endothelial cells [140-141]. Apart from non-phagocytic cells, cellular uptake of these particles has also been reported for phagocytic cells. A study by Costantin et al. [23]
reported the uptake mechanism of engineered nanoparticles in macrophages via phagocytosis. However, particle size-dependent phagocytosis has not been reported for engineered silica particles as observed for other types of engineered particles such as titanium oxide [142], quantum dots [143], and gold nanoparticles [144]. Careful assessment of all the particle parameters in the induction of not only inflammatory response but also other cell-particle interaction events, such as the internalization mechanism, would allow for in vitro cell culture systems to be used as screening tools for newly-synthesized particles. It is optimal to collect data at different stages of particle-cell interaction to develop a series of toxicity assays that can limit the demand for in vivo studies, both from a cost, time as well as animal-use perspective.

A secondary aim of this investigation was to visualize the macrophages when exposed to a high cytotoxic dose as compared to a non-cytotoxic dose that is relevant to environmental exposure levels of natural silica particles. This is important to understand as currently one of the main caveats of in vitro toxicological studies is the unrealistically high dose of 100 μg/ml or more that is typically used in these studies. Understanding the effects of high cytotoxic doses during all aspects of cell-particle interaction from cellular uptake to inflammatory response would allow for the design of efficient and controlled in vitro systems for a given risk-exposure scenario.

4.3 Materials and methods

4.3.1 Materials and reagents

Aclar sheets, Poly/Bed 812® resin, propylene oxide, and embedding capsules were purchased from Ted Pella. Cerium chloride, lead citrate, uranyl acetate, and osmium tetroxide were
purchased from Sigma-Aldrich. All the chemicals required for the 50 nm silica synthesis were also purchased from Sigma-Aldrich.

4.3.2 50 nm silica synthesis

50 nm silica particles were synthesized using the Stöber process [145-147] by dissolving 0.75 ml of 29% ammonium hydroxide in water to 14.29 ml anhydrous ethanol and stirred for 5 minutes. After mixing, 0.375 ml of Tetraethyl Orthosilicate (TEOS) was added dropwise under vigorous stirring, and stirred continuously for 4 hours. The resultant particles were then collected by centrifugation (12,000 rpm for 10 minutes), washed with water, followed by multiple washes with ethanol and dried at 60 °C for 12 hours.

4.3.3 Particle characterization

Detailed characterization of the 50 nm silica particles is described in Chapter 3 under section 3.4.1.

4.3.4 Electron microscopy study

For TEM sample preparation, the cell culture plates were lined with aclar sheets and THP-1 monocytes (2 x 10^6 cells) were added to each well to undergo differentiation with 50 ng/ml PMA. Following which, cells were treated with 1 and 100 µg/ml of 50 nm silica nanoparticles for 24 hours. Cells were then fixed with 2.5% glutaraldehyde in Phosphate Buffered Saline (PBS), pH 7.4 for 2-3 hours at room temperature and then transferred to 4 °C for 24 hours. Excessive glutaraldehyde was removed and cells were washed with PBS buffer for 2-3 times. The culture dishes were then post-fixed with osmium tetroxide in PBS buffer for 10 minutes at room temperature and transferred to 4 °C for 24 hours. The osmium tetroxide was then suctioned off and the culture plates were first washed with PBS for 2-3 times and then dehydrated at 50 %,
75 %, 95 %, and 100 % ethanol (30 minutes each dehydration). The cells were then embedded by using a mixture of resin and propylene oxide in different series (1:1, 3:1 and 100 % resin for 30 minutes each). The embedding capsules filled with resin were then inverted and pressed till the resin was in direct contact with the aclar sheets. The sample and the resin in the capsule were then heated in a 60 °C oven for 24 hours in order for the resin to polymerize. Next, the resin block was removed from the aclar sheets by using liquid nitrogen and subjected to thin sectioning using an ultramicrotome. Ultra thin sections (below 100 nm) were cut and placed on copper grids, and then post-stained with uranyl acetate and lead citrate and examined by TEM.

4.3.5 Optical microscopy study

The 2 μm silica suspension was prepared in 1X PBS and dispersed by a sonicator to avoid aggregation. The suspensions were freshly prepared, diluted, and then immediately treated to differentiated THP-1 macrophages for 24 hours. After 24 hours, the cells were imaged with a Nikon C1 confocal microscope.

4.4 Results and Discussion

SEM micrographs of the 2 μm silica particles showed uniform, spherical particles with a standard deviation of +/- 0.18 μm measured by counting 400 particles in the micrographs (Table 1, Figure 1A; chapter 3). The TEM micrographs of silica nanoparticles, synthesized using the Stöber process with no calcination, rendered fairly spherical particles with a mean size of 50 nm with standard deviation of +/- 20 nm. The TEM micrographs also indicated some degree of aggregation in the nanoparticles (Figure 1). Some of the aggregates consisting of two or three particles were fused together but were judged to be still in the nano range as observed in the micrographs. These aggregates are a common occurrence during the synthesis of smaller
particles as compared to micron-sized particles. The phase of the 2 μm and 50 nm particles was amorphous, as clearly shown from the X-ray Diffraction (XRD) broadened peaks and diffuse or halo electron diffraction pattern (Chapter 3, Figure 1A c).

Alveolar macrophages carry out a major pulmonary defense mechanism to protect the lungs by phagocytizing particles [148]. In the presence of 2 μm particles, the macrophages phagocytosed by extending their pseudopods outwards and engulfing some of the particles in membrane bound compartments called phagosomes (Figure 2). However, several particles were observed either surrounding the macrophage membrane or in membrane-bound phagosomes at the macrophage membrane. On the other hand, TEM observation of the uptake of 50 nm silica particles showed more nanoparticles being actively phagocytosed and aggregating inside the phagosomes into nano-clusters (dense, high contrast). This process was observed for other small mineral particles, which tended to end up aggregated inside the phagosomes and lysosomes even if they were taken up as individual particles [149]. The fusion of the phagosomes, containing the silica particles, with the lysosomes that have degrading proteolytic enzymes to digest the particles, was also observed (Figure 3A, B, C, G). These findings correlated with previous studies involving silica and other engineered particles that indicated engineered particles being uptaken into cells primarily by phagocytosis [23, 141, 150]. However, a few isolated nanoparticle clusters were also observed inside the macrophage cytosol, suggesting some interruption or irregularity in the phagocytosis mechanism by the macrophages and involvement of other uptake mechanisms (Figure 3B). Similar findings were also observed by Geiser et al. 2007 [151] during the phagocytosis of titanium oxide nanoparticles. The evidence that nanoparticles can bypass the most important clearance mechanism, namely phagocytic uptake of macrophages, requires further clarification as to whether these results were specific for the size,
phase or other particle parameters. The consequence of this non-phagocytotic uptake, however, could suggest direct contact of the nanoparticles with the cellular organelles leading to increased toxicity and other cellular response, such as DNA damage.

The size effect on phagocytosis was examined by observing the optical and TEM micrographs of the 2 μm and 50 nm engineered silica particle internalized by the macrophages. The relevance of this aim was to not only compare the ability of micro- and nano-particles to induce inflammation (as observed in chapters 2 and 3), but also to understand their effect on alveolar macrophage clearance functions of phagocytosis. The cellular uptake appeared particle size-dependent in the order of 50 nm > 2 μm. This is consistent with other studies that demonstrated quantitatively that the optimum size for cellular uptake was 50 nm for other type of particles[152]. A study by Osaki et al. [143] and, Jiang et al. [144] reported that 50 nm glycovirus and 50 nm gold nanoparticles entered cells via receptor-mediated phagocytosis more effectively than other smaller and larger particles respectively. Jiang et al. [144] hypothesized and attributed the efficient uptake of 50 nm gold particles to the internalization process being strongly dependent on the cell membrane wrapping time, which is based on the clustering of the receptors present on the cell membrane surface. Smaller nanoparticles could dissociate from the receptors before being engulfed by the membrane owing to low binding affinity, whereas, extremely large particles tend to bind to several receptors at the same time, which extends the membrane wrapping time that is necessary for nanoparticle internalization [153]. The 50 nm particles appeared to be the critical cutoff point for receptor-mediated internalization. This study determines that not only the silica-induced pro-inflammatory mechanism, observed in chapters 2 and 3, but also the initial internalization of the particles by the macrophages is particle size-dependent.
The secondary aim of this work was to observe the cellular environment as a consequence of a high and low particle dose. Since, the relevance of this dissertation work was to conduct experiments with particle doses that are more relevant to real-life exposure situations (chapters 2 and 3) and not high cytotoxic doses. Here, at a low non-cytotoxic, realistic dose of 1 µg/ml, the macrophages were not overwhelmed with the engineered micro- and nanosilica particles and showed no morphological changes (Figures 2C, 3H). This could result in the activation of the pro-inflammatory mechanism discussed in chapters 2 and 3. Upon interaction of the particles containing iron with the phagocytic receptors in the plasma membrane region signal transduction occurs by the activation of NADPH oxidase enzyme. This results in the production of superoxide, and thus, hydrogen peroxide. Further, mediation of the inflammatory response could be the direct result of lipid raft disruption via PC-PLC activation. However, at a higher cytotoxic, unrealistic micro- and nano-silica particle dose of 100 µg/ml (Figures 2C, 3C), cells appeared overwhelmed with the particles and showed some morphological changes, such as an increase in the number of lysosomes, indicating an enhanced macrophage clearance mechanism. The number of particles per cell was increasingly high, with some cells completely covered with particles (shown with a red arrow Figure 2C). This indicated "particle overload", which could change the macrophage function in conducting cell response, such as depression in the particle clearance mechanism leading to increases in the inflammatory cell influx in the lungs [149]. Persistent inflammation due to macrophages being overloaded with particles could lead to the trigger of cell injury events, such as necrosis, resulting in the release of pro-inflammatory molecules from the dying cells, thus exacerbating the overall inflammatory response [154]. Also, if the strong degrading enzymes of the lysosomal compartment of the macrophages are unable to break down the particles after internalizing them, it could result in a loss of membrane
integrity. Some investigators believe that the failed attempt at silica digestion by the macrophage leads to caspase (essential proteins for cell death) activation-induced apoptosis leading to inflammatory response [39]. Excessive release of inflammatory mediators through such events would make it difficult to isolate and study the signaling responses caused by nanoparticle-induced inflammation alone. The accumulation of the particles due to the failure of the macrophage clearance mechanism, as well as cell debris build up, and excessive inflammatory response could ultimately cause lung overburden leading to lung fibrosis [155]. This indicated the importance of in vitro and in vivo studies using realistic exposure levels [51, 148]. The mechanism beyond phagocytosis of the particle has been discussed in detail in chapters 2 and 3. The study in this chapter suggested that cellular uptake of particles in macrophages was predominately by phagocytosis and was size-dependent, although some nonphagocytotic uptake of the 50 nm silica particles appear to occur as well. Furthermore, this study also portrayed the need to use a low non-cytotoxic dose, as in the work in chapters 2 and 3, in studies demonstrating differences in particle-induced cytotoxicity and other cellular response such as inflammation.
4.5 Figures and figure legends

Figure 1: Representative micrograph of the synthesized 50 nm silica particles. The silica nanoparticles looked fairly spherical with but showed some degree of aggregation as indicated by the arrow.
Figure 2: 2 µm silica particles uptake by THP-1 macrophages. 2 µm silica particles were actively uptaken by the macrophages, mostly around the membrane periphery and some in the cell cytosol. Macrophage over burden: excess phagocytosable particles, can be observed when cells were treated with 100 µg/ml (C) of silica particles as compared to 1 µg/ml (B) of silica concentration, where cells are not saturated with particles. White arrows indicate silica particles. Red arrow indicates macrophage overload.
Figure 3: 50 nm silica particle uptake by THP-1 macrophages at different stages as studied by TEM. (A) Depicts numerous vacuoles called phagosomes with nanoparticle aggregates encapsulated within them indicated by the arrow, (B) Few particles appeared to be isolated without any phagosome encapsulation, (C) Depicts particles inside large vacuole formation after the fusion of phagosomes with lysosomes, (D) Silica nanoparticle-cell membrane surface
interaction can be observed in this micrograph, (E) and (F) High magnification micrograph to observe the vacuoles closely after low concentration of 1 µg/ml exposure showed dispersed particles in the macrophage, (G) Depicts the process of fusion of lysosome with phagosome containing particles, and (H) Shows the interaction of the macrophage pseudopods with silica nanoparticles
Inhalation of iron contaminated natural crystalline silica induces respiratory diseases. However, the pro-inflammatory mechanism by which the iron on natural silica particles cause disease is not conclusive due to variability in the source of origin combined with incomplete characterization, control, and use of high particle doses. The work in this study determined a novel mechanism of characterized silica particle-induced pro-inflammatory increase in the presence of iron at a dose closer to the realistic exposure regime. Natural silica particles in the presence and absence of iron (acid treated) were characterized for size, iron, phase, and surface area. Iron was found to be mediating lipid peroxidation and lipid-raft disruption leading to inflammatory cytokine production through PC-PLC in silica-exposed macrophages at a low non-cytotoxic dose of 1 μg/ml. This was determined by increases in superoxide, lipid peroxidation, lipid raft disruption, and cytokine production upon treatment with natural silica containing iron and decreases of these responses upon treatment with iron chelators DTPA and DFO. Activation of PC-PLC in this mechanism was confirmed by using tricychodecan-9-yl-xanthate, a competitive inhibitor of PC-PLC, which inhibited silica-stimulated cytokines. This observed novel pathway clarified some of the ambiguities in the understanding of silica-induced pro-inflammatory mechanism involving iron at doses closer to real-life situations. The key role of iron shown here suggested that by reducing iron on the surface of particles, there is a possibility of mitigating the respiratory effects of air-borne particles exposure.

However, due to the inhomogeneity (with respect to composition, morphology and size) of the naturally occurring silica particles, it was difficult to independently analyze the effects of
iron alone on the novel pathway of iron-mediated silica induction of inflammatory cytokines through PC-PLC observed by the work in chapter 2. Therefore, engineered silica particles were introduced to investigate the above-mentioned inflammatory mechanism. Engineered silica particles are better-controlled and more homogenous in size, shape, and surface chemistry than natural silica particles. Furthermore, with the increase in use of engineered nanoparticles for different applications, such as drug delivery, and increasing evidence suggesting that engineered nanosilica particles could also contribute to the production of reactive oxygen species as observed with natural silica, it was also important to analyze the pro-inflammatory induction due to exposure to a low, non-cytotoxic dose of engineered nanosilica. Therefore, this work was studied using micro- (2 μm) (same size as natural silica) and nano-sized (50 nm) engineered silica particles. Even though these particles are more homogeneous, they can still be variable depending on their source of origin, so thorough characterization needs to be carried out before analyzing them. Both particle sizes were iron-doped and characterized for size, morphology, phase, surface iron, and aggregation. This work demonstrated that iron-doped 50 nm particles induced elevated levels of superoxide, lipid peroxidation, and cytokine (IL-1β and TNF-α) production in the macrophage membrane, through PC-PLC, compared to 50 nm undoped particles, as well as iron-doped and undoped 2 μm particles at 1 μg/ml. This emphasizes the role of not only iron, but also particle size in the silica-induced pro-inflammatory mechanism studied in this work. Upon comparing the work from chapters 2 and 3 it was also concluded that particle phase could possibly play a role as well in the pro-inflammatory mechanism induced by these particles, since 2 μm natural crystalline particles showed a greater cellular response than the 2 μm engineered amorphous silica particles in the novel mechanism investigated in this dissertation work. The work with controlled silica particles further emphasized that iron traces
on silica particles play an important role in triggering the pro-inflammatory mechanism induced by these particles. It also demonstrated that not only natural silica particles, but also engineered silica particles, act through the same pro-inflammatory pathway.

Lastly, the effect of particle size and particle dose on the cellular uptake mechanism of the macrophages was investigated by treating macrophages with engineered micro- and nanosized silica particles at a low and high dose. This study was performed to understand if particle size and use of a high dose affected inflammation only or whether this affected the initial internalization mechanism as well. It was visualized microscopically using optical microscopy and TEM that the macrophages via phagocytosis took up the particles in a size-dependent manner, with the internalization being greater for nanoparticles. However, some isolated particles were found in the cell cytosol suggesting a nonphagocytic mechanism, which has been observed with other types of particles as well. Furthermore, microscopy studies allowed observation of the cell environment when exposed to a high dose as used in previous studies. It was illustrated that, at a high cytotoxic dose of 100 μg/ml, macrophages were overburdened with particles, which suggested possible alterations in macrophage clearance which could affect the pro-inflammatory mechanism investigated in chapters 2 and 3. In summary, this study indicated that it was crucial to identify, characterize and control the different particle parameters before testing for particle-induced cellular interactions such as toxicity and inflammation.

There are several areas for future work. In vitro cell cultures are emerging as screening tools for nanoparticle toxicity and inflammation, but it is important to identify the most relevant type of in vitro system under controlled conditions for risk assessment. An in vitro co-culture system would enable an in-depth investigation of how cell-cell interactions in the lung environment would behave upon exposure to doses similar to a realistic exposure setting. It
would also be relevant to study different exposure scenarios based on mass, surface area and particle number to more accurately understand the risk-exposure scenario. With the growth in nanotechnology, newer nanoparticles are produced everyday for various applications. Development of monitoring techniques to check for the other relevant particle parameters, such as particle morphology, particle functionalization, and other particle sizes at realistic doses that trigger the signaling pathway causing inflammation as shown by the study here will help develop regulatory measures for nanoparticle exposure.
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