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TITLE

Amorphous Nanosilica induced Toxicity, Inflammation and Innate Immune Responses: A Critical Review

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HIGHLIGHTS

- Toxic effects of Amorphous nanosilica
- Amorphous nanosilica induced cytotoxicity and altered biological functions in humans
- Particle size, surface and concentration dependent cytotoxicity effects
- Amorphous nanosilica commercialization and safety concerns

ABSTRACT

Nanoparticles are promising bioengineering platforms facilitating various consumer product formulations, including packaged food, electrical, biosensors and biomedical tools. The unique surface and physicochemical properties of amorphous nanosilica supports advanced nano-biomolecular applications for various manufacturing, biotechnology, and healthcare industries including cosmetics, packaging, implants, drug delivery systems and cancer diagnostics. The increased technological and economic benefits of amorphous nanosilica, raises concerns regarding their adverse biological effects on humans. The cellular mechanisms underlying amorphous nanosilica internalization, evasion of biological barriers, inadvertent nano-bio interactions and unexpected long term exposure effects must be taken into consideration from the diverse ecosystems and human safety aspects. Recent research studies reveal cytotoxic, inflammatory and immunomodulatory effects of amorphous nanosilica particles. Our review focuses on studies demonstrating hazardous impact of amorphous nanosilica/bio-systems interface on the cellular and biochemical processes. The review further seeks to evaluate amorphous nanosilica-induced cytotoxicity, innate immune responses, inflammation and immune related dysfunctions, and discuss open research questions related to the use of amorphous nanosilica in biomedicine.

KEY WORDS

Amorphous silica nanoparticles, Cytotoxicity, Inflammation, Inflammasome, Immune system, Toxicity

1. INTRODUCTION

Nanoparticles are promising bioengineering platforms facilitating various consumer product formulations including cosmetics, biosensors and cancer diagnostic tools. Nanomaterials tend to acquire novel physical, chemical and surface characteristics distinct from the parent material. They easily invade our skin, vital organs, blood vessels and can even surpass the blood-brain barrier(Lockman et al. 2004; Wohlfart et al. 2012). Advances in nanotechnology have made possible effective manipulation and regulation of specific biological mechanisms at the range of nanoscale. Silicon dioxide (also known as silica) is one of the most abundant elements on earth, present in both crystalline and amorphous forms. Crystalline silica or silica dust has serious health hazards associated with occupational exposure, such as mines and construction industries. It is also the most common toxic inhalable particulate matter, inducing inflammation, toxicity and altered immunological responses in humans(Napierska et al.). Several epidemiological studies have shown inflammatory and carcinogenic effects in humans under a wide variety of silica exposure including mines and industries(Kachuri et al. 2014; Lee et al. 2016; Liu et al. 2020; Mehri et al. 2020; Scalia Carneiro et al. 2020; Tse et al. 2011; Vida et al. 2010). Crystalline silica exposure is linked to several inflammation-associated diseases including pneumoconiosis, silicosis, lung cancer and auto-immune disorders(Pollard 2016; Steenland and Sanderson 2001). In contrast, amorphous nanosilica exhibits unique surface and physicochemical properties. Amorphous nanosilica support extensive nano-biomolecular applications with enormous potential impact in the field of material science, manufacturing, biotechnology, food, medicine, diagnostics and healthcare industries(Barik et al. 2008). However, recent research studies bring amorphous silica under scrutiny due to its toxic biological effects, raising human safety concerns about routine exposure that mandates clinical evaluation(Mebert et al. 2017).

Although respirable nanosilica is the most common mode of amorphous nanosilica exposure, the rapid pace of commercialization leaves the entire human system prone to nanosilica-induced toxicity, abrupt epigenetic, phenotypic and immune system-related alterations (Figure 1). Contrary to the various well-understood mechanisms by which crystalline silica induces persistent inflammation, relatively little is known about the adverse health effects caused by amorphous silica exposure (Peeters et al. 2013; Perkins et al. 2016; Tsugita et al. 2017b; Yazdi et al. 2010). Our review presents an overview of evidence that highlight the harmful effects of amorphous nanosilica in humans and safety concerns related to the promising bio-medicinal aspects of amorphous silica.

2.1 Amorphous Nanosilica-induced Toxicity: Particle Concentration, Exposure and Cell Type Dependent

Amorphous silica nanoparticles (SiNPs) can enter the bloodstream through multiple routes of exposure, playing a vital role in drug delivery and cancer therapy (Figure 1). Studies on SiNPs demonstrating their structural and functional particle stability, cellular internalization, nanosilica-induced inflammatory signaling, immunomodulation and long term toxicity effects are scarce. The cell signaling mechanisms employed for these unprogrammed nano-bio interactions occurring *in vivo* between SiNPs and biomolecules residing in the intracellular milieu are unclear. Recent studies have shown amorphous nanosilica as potent inducers of toxicity and inflammation in humans(Di Cristo et al. 2015; Nabeshi et al. 2011a; Napierska et al. 2013). Amorphous nanosilica induced toxic responses are cell-specific, and increase with increasing particle concentration and exposure time (Table 1). Nanosilica size acts as an important parameter that determines the SiNPs' potential to induce cellular response(Wang et al. 2018). SiNPs-induced cytotoxic response and cell death increase with increased particle concentration

and decreased particle size(Wang et al. 2018),(Guo et al. 2016). In the last few years, several researchers have shown size, concentration and cell type-specific effects of nanosilica. A study by Decan *et al.*, reports concentration and time-dependent genotoxic, cytotoxic and transcriptomic responses of nanosilica in mouse lung epithelial (FE1) cells. After 24 hours, small-sized amorphous silica particles (12nm and 5-15nm; 100 μg/ml) caused significant loss in cell viability as compared to large-sized silica (10-20nm and 2μm). Additionally, 12nm or 2μm silica exposed cells (12.5μg/ml and 50μg/ml) show disrupted cellular responses including increased reactive oxygen species (ROS) and micronucleus formation(Decan et al. 2016). Also, 12nm nanosilica exhibited significantly high cell death rate. The long-term effects (12.5 μg/ml; 7 days) assessed for cellular proliferation capability, included significantly reduced cell survival in response to 12nm silica(Decan et al. 2016). Amorphous nanosilica induced damage to intracellular structures, fragmented nuclei and cytosol destruction has been observed in transmission electron miroscopic (TEM) image sections of 20nm SiNPs treated epithelial (A549 and HepG2) and fibroblast (NIH/3T3) cells(Kim et al. 2015). Similarly, 15 nm and 46nm amorphous silica caused high cytotoxicity in human bronchoalveolar carcinoma cells as compared to crystalline silica in a dosage (10-100μg/mL) and time (24-72 hours) dependent manner (Lin et al. 2006). The above mentioned studies reveal amorphous nanosilica is more toxic as compared to the crystalline silica.

Napierska et al., observed the toxic effects of amorphous nanosilica in endothelial cells(Napierska et al. 2009). Breznan *et al.* group observed cell-type, dose and particle-dependent toxic effects of different-sized nanosilica in human lung epithelial cells (A549), human monocytes (THP1) and mouse macrophages (J774A.1)(Breznan et al. 2017). Interestingly, macrophages were more susceptible to nanosilica as compared to other cell types. Several studies

have shown high ROS production and subsequent increase in K^+ efflux and intracellular Ca^{2+} levels by crystalline silica(Ahmad et al. 2012; Hegde et al. 2018; Hornung et al. 2008; Muñoz-Planillo et al. 2013; Rossol et al. 2012). Peilin and Jin *et al.*, recently reported novel bi-directional regulation of amorphous SiNPs (10-20nm) induced toxicity in HEK293 cells by differential expression of TRPM2 channels. SiNPs-induced ROS production causes TRPM2, a transient receptor potential Melastatin 2 plasma membrane channel activation and subsequent loss of cell viability. TRPM2 activation upregulates NOX2 and increases NADPH oxidase activity, a positive feedback for ROS generation and intracellular Ca^{2+} increase causing severe cell death in the TRPM2-LE (low expression) cells. Whereas, TRPM2 activation in the TRPM2-HE (high expression) cells downregulate NOX4 expression to inhibit NADPH oxidase activity and alleviate nanosilica induced cytotoxicity(Yu et al. 2015). High TRPM2 expression makes senescent mice macrophages less susceptible to nanosilica exposure as opposed to young mice. The findings reflect age-dependent nanotoxicity, suggesting both detrimental and protective functions of TRPM2 in many physiological and pathological processes. Surprisingly, amorphous nanosilica also causes endothelial cell damage, platelet activation and hyperactivation of the coagulation system. Nanosilica elicits significant apoptosis in human umbilical vein endothelial cells (HUVECs) mediated via p53-caspase (-9,7,3) pathway(Wang et al. 2018). Guo *et al.*, revealed nanosilica-triggered autophagy and apoptosis in endothelial cells via ROS-mediated MAPK/Bcl-2 and PI3K/Akt/mTOR signaling pathways(Guo et al. 2016). Amorphous nanosilica exposure causes anti-coagulation factor depletion and fibrinolytic resistance in Wistar rats via coagulation factor XII and JNK-NF-κB/AP-1 pathway activation(Jiang et al. 2015). *In vivo* TEM images of histopathological analysis depict SiNPs (30nm, 70 nm) present in liver and brain, after

getting absorbed through the nasal cavity of the BALB/c mice. SiNPs absorption triggers the coagulation cascade via coagulation factor XII activation in plasma(Yoshida et al. 2013).

Increased commercialization of amorphous nanosilica-based applications has left humans exposed to raw and engineered silica nanoparticles. Lungs and liver remain one of the main targets organs of nanosilica exposure in humans. A study by Li *et al.*, explores the cellular mechanisms underlying nanosilica internalization and report subcellular SiNP distribution, ultrastructure damage, and cytotoxicity in human hepatic (L-02) cells(Li et al. 2016). TEM images suggest nanosilica internalization (46 and 64nm) occurs in the cytoplasm through endocytosis and diffusion. Nanosilica content was high in the 46nm SiNPs-treated group. Nanosilica treatment causes structural mitochondrial dysfunctions including vacuoles formation, swollen and disrupted mitochondrial cristae, degranulated endoplasmic reticulum, damaged lysosomal vesicles and autophagolysosomal structures. Further scanning electron microscopy (SEM) analysis, shows a large number of SiNPs adhering to the cell surface. Increased nanosilica dosage significantly decreases SOD and GSH-PX activity indicating low anti-oxidant activity, accompanied by increased LDH levels and MMP loss suggesting SiNPs-induced membrane and mitochondrial injury through oxidative damage. Both 64nm and 46nm SiNPs could induce apoptosis and necrosis but 46nm nanosilica induced extensive oxidative damage and cytotoxic effects in L-02 cells(Li et al. 2016).

Despite similar primary particle size, SiNPs show distinct cytotoxicity and inflammatory profiles across species and cell types. A study by Yazdi et al. shows how keratinocytes (unlike the myeloid cell response to micro-sized silica) phagocytize SiNPs (15nm) leading to inflammasome activation and IL-1β, cytokine release. However, TiO_2 NPs (20nm, 80nm) enter THP1 (human monocytic) cells through phagocytosis-independent pathways. Our recent research shows size,

concentration and exposure time-dependent effects of amorphous nanosilica on human bronchoalveolar epithelial, endothelial and fibroblast cell lines. Results reveal distinct pathways for nanosilica-induced cytotoxicity in each cell type. While 22nm nanosilica induces apoptotic cell death, the 12nm nanosilica induces necrotic cell death. Nanosilica uptake is actin cytoskeleton dependent in 22nm and independent for 12nm size particles. Apoptosis-associated speck-like protein (ASC) expression and specks formation increases in response to 12nm nanosilica (Sharma *et al.*, unpublished data). *In vitro* results show significant cytotoxicity in A549 cells for bare SiNPs and amine-functionalized SiNPs at a higher concentration (200 mg/mL) for 24 - 48 hour exposure(Morris et al. 2016). SiNPs-exposed human erythrocytes undergo oxidative damage and reduced ATPase activity, which ultimately leads to hemolysis(Jiang et al. 2016). Similar observations made by Nemmar *et al.*, reveal SiNPs (50nm) induce a dose-dependent hemolytic effect and apoptosis in mouse erythrocytes *in vitro*. Further, confirmed by gradual increase in LDH release, SOD and catalase activity (anti-oxidants), cytosolic Ca^{2+} concentration and reduced GSH levels in nanosilica treated erythrocytes(Nemmar et al. 2014). Recently, Park *et al.* reported nanosilica of size- 50nm, 100nm and 150nm (100μg/ml; 48 hrs) did not induce significant cytotoxicity in cultured human corneal epithelial cells (HCECs). Interestingly, the intracellular survival mechanisms, autophagy and mammalian target of rapamycin (mTOR) pathway remained intact in nanosilica treated cells. However, SiNPs did get internalized and localized into the cytoplasmic vacuoles of HCECs(Park et al. 2016). The effect of particle size on the SiNPs and cellular interactions was also studied using two different sized SiNPs (64nm, 46nm) at 50 μg/mL concentration. Using ICP-AES (Inductively coupled plasma atomic emission spectroscopy), significantly elevated SiNPs levels were detected with increasing particle concentration(Li et al. 2016). A study by Vis et al., shows

the ultrasmall silica nanoparticles (3.6–5.1 nm in diameter) induce dose-dependent CD4 and CD8 T-cell activation through cell surface CD25 and CD69 up-regulation at concentrations above 150 μM Silica. Given the routine human exposure to ultrasmall particles and their emerging bioclinical applications, their off-target immunomodulatory effects via direct T-cell activation should be carefully considered(Vis et al. 2018).

Classically activated macrophages/M1 macrophages mediate host defence against various pathogens and also mediate anti-tumor immune responses. Alternatively activated macrophages/M2 macrophages have an anti-inflammatory function regulating tissue repair and suppression of M1 responses(Murray and Wynn 2011). Interestingly, M2 polarization of macrophages promotes nanoparticle internalization both *in vitro* and *in vivo*. At a concentration of 50μg/ml, nanosilica uptake was higher for M2-polarized as compared to M1- human monocyte-derived macrophages and THP-1 cells. Similarly, M2-like primary human tumor-associated macrophages (TAM) obtained from lung tumors endocytosed more SiNPs. Surprisingly, the uptake of microsized silica was macrophage phenotype-independent(Hoppstädter et al. 2015). The co-exposure of polyethylene glycol (PEG)-coated SiNPs (90nm) during ovalbumin (allergen) sensitization, causes a dose-dependent increase in allergic airway disease manifested by significantly high OVA-specific serum IgE, eosinophil infiltration, and Th2 and Th17 cytokine expression. Due to these adjuvant-like properties of nanosilica, SiNPs exposed individuals might have increased allergen sensitization and are more likely to manifest allergic airway disease(Brandenberger et al. 2013). Collectively, all these findings demonstrate amorphous nanosilica induced toxicity is not only cell specific but also particle concentration and exposure time-dependent (Table 1).

2.2 Carcinogenic potential of amorphous Nanosilica

Researchers provide evidence for the carcinogenic effect of amorphous silica nanoparticles. Gonzalez et al., demonstrate direct effects of SiNPs leading to reduced microtubule dynamics, microtubule acetylation and cell migration capacity of interphase human lung carcinoma cells (A549)(Gonzalez et al. 2015). Guo et al., observed chronic low dose SiNPs exposure resulted in aberrant p53 signaling and subsequent malignant transformation of human lung epithelial cells (BEAS-2B) indicated by enhanced cell growth, proliferation and migration. Additionally, the transformed cells were able to induce tumorigenesis in nude mice(Guo et al. 2017). SiNPs transport across the fetal compartment in choriocarcinoma cells (BeWo b30) and *ex vivo* perfused human placenta, questions the biocompatibility and rapid expansion of nanosilica applications in bioimaging, medical implants and therapy(Poulsen et al. 2015). Chronic SiNPs (45nm) exposure by tracheal perfusion for 15 days induced female reproductive dysfunction in mice(Liu et al. 2018). Liu *et al.*, observed ovarian damage, sex hormones imbalance, increased formation of atretic and primary follicles, followed by oxidative stress and DNA strand breaks (day-15). These clinical manifestations are reflected by a significant increase in ATM, CHK-2, P53, E2F1, P73, BAX, Caspase-9 and Caspase-3, and downregulation of RAD51 protein expression which assists ds-DNA breaks repair. Increased oxidative stress and severe DNA damage could be inducing mitochondria-mediated apoptosis of granulosa cells. Interestingly, SiNPs-induced cellular damage and toxicity recovered by 30 days, this shows SiNPs cause reversible damage to follicles in mice(Liu et al. 2018). Emerging research evidence suggest the carcinogenic potential of amorphous SiNPs, questioning the biocompatibility and rapid expansion of nanosilica applications in bioimaging and cancer therapeutics.

2.3 Amorphous Nanosilica-Induced Inflammasome Activation

Innate immunity adapts strong defense mechanisms against a specific pathogen or damage-associated stimuli. Activation of NLRs, a highly conserved class of cytosolic pattern recognition receptors is one of the defense mechanism that is activated. NLRs are nucleotide binding domain, leucine-rich repeat-containing receptor proteins serving as critical mediators of innate immune regulation in humans. Dysfunction in NLRs is linked to a wide spectrum of inflammatory diseases, including silicosis, gastric and metabolic disorders, pulmonary dysfunctions, inflammatory bowel disease and cancer(Allen 2014; Davis et al. 2011; De Nardo and Latz 2011; dos Santos et al. 2012; Sharma and Jha 2017). Each of the NLR family members is activated by diverse series of specific endogenous and exogenous stimuli. Some of the activated NLRs form large multiprotein complex platforms, known as inflammasomes (Figure 2). NLR upon activation, recruits ASC (Apoptosis-associated speck-like protein containing CARD) adaptor molecule followed by the auto-proteolytic cleavage of pro-caspase-1 into caspase-1, an active cysteine protease enzyme(Dick et al. 2016; Franchi et al. 2009). Caspase-1 then cleaves one of its substrate pro-IL-1β into its active IL-1β form(Keller et al. 2008). Recently, scientists have discovered caspase-1 self-cleavage, as an intrinsic mechanism to terminate inflammasome activity(Boucher et al. 2018). Therefore, inflammasome activation regulates the transcription and release of potent pro-inflammatory cytokines, IL-1β and IL-18, and subsequent activation of major inflammatory and cell death-associated pathways(Martinon et al. 2002).

Research advances have provided biomedical implants for initiating repair or restoring biological tissue functionality. However, often implantable biomaterials induce foreign body response, leading to an uncontrolled array of inflammatory reactions, modulated host immune cell phenotypes and functionality. Therefore, the surface topography and chemistry of implant

materials should be utilized carefully for exerting control over predictable inflammatory and immune responses. The evidence suggests potential role of inflammasome components in modulating immune responses upon biomaterial implantation, as nano-debris derived from implants trigger NLRP3 inflammasome activation. In this aspect, Christo *et al.*, found potential involvement of inflammasome components in altered macrophage responses to controlled nanotopography surface and chemistry. *ASC*$^{-/-}$ and *NLRP3*$^{-/-}$ bone marrow-derived macrophages (BMDMs) in contrast to *AIM2*$^{-/-}$ BMDMs, show low IL-1β, high IL-6 and TNF-α levels, reduced cell adhesion, when cultured on a substrate with controlled surface topography(Christo et al. 2016). Shirasuna *et al.*, have found the novel pathophysiological role of ASC and NLRP3 inflammasome activation in pregnancy. Nanosilica (70nm) -induced placental inflammation and pregnancy complications was reduced in *Nlrp3*$^{-/-}$ mice and completely prevented by ROS inhibition or IL-10 overexpression in mice(Shirasuna et al. 2015).

Dendritic cells (DCs) and macrophages are important regulators of intestinal immune homeostasis, critical for the induction of both innate and adaptive immune responses against pathogens or foreign antigens. The immunogenic potential of amorphous nanosilica, in terms of DC activation has been analyzed *in vitro* using murine BMDCs. Amorphous SiNPs, crystalline silica and TiO_2 NPs directly stimulate DCs by upregulating MHC-II, CD80 and CD86 expression, followed by inflammasome activation and subsequent IL-1β release in wild type but not *Caspase-1/Nlrp3* deficient mice(Winter et al. 2011). Similarly, Malachin *et al.*, observed synergistic effects of commensal bacteria and amorphous SiNPs in the DCs. Thus, dendritic cells are indispensable for host-microbe balance in tissues such as gut and skin, likely exposed to nanoparticles present in drugs, food and additives. SiNPs (<10-20nm) cause dose-dependent decrease in cell viability by inducing oxidative stress and pro-inflammatory responses in L-132,

human lung epithelial cells(Sahu et al. 2015). Peeters *et al.* group further demonstrated NLRP3 inflammation in SiNPs treated human bronchial epithelial cells (BEAS-2B). Interestingly, Caspase-1 and NLRP3 inflammasome activation led to IL-1β release, and a significant increase in the unconventional release of the alarmins bFGF and HMGB1(Peeters et al. 2013). Individual SiNPs (30nm) and TiNPs (21nm) immediately form aggregates; stable and monodisperse complexes of size ~ 250nm in presence of divalent cations(Tsugita et al. 2017a). Together, SiO_2 and TiO_2 NPs at lower concentrations synergistically trigger significantly high Caspase-1 activation and IL-1β release in mouse bone marrow-derived macrophages and lung inflammatory responses when mice treated intratracheally(Tsugita et al. 2017a). Individually, SiO2and TiO2 NPs induce different cellular stress pathways in BMDMs, as SiNPs localized into lysosomal compartments but TiNPs do not. Instead, TiNPs induce high levels of ROS production as opposed to SiNPs.

Interleukin-1β (IL-1β), a pro-inflammatory cytokine propagates nanosilica-induced inflammation by promoting granulocyte migration towards the affected sites(Groß et al. 2012; Guo et al. 2013; Martinon et al. 2006). IL-1β positively autoregulates its synthesis by specific functional NF-κB binding site activation(Hiscott et al. 1993). Alarmins are a distinct class of endogenous mediators passively released from the necrotic cells or stimulated leukocytes in response to any sterile injury or infection. Alarmins perform important intracellular roles to maintain cellular homeostasis by promoting leukocyte recruitment and tissue repair upon extracellular release(Yang et al. 2017). Contrary to IL-1 β, both IL-1α and IL-33 are synthesized as active precursor-forms. During necrosis, IL-1α, IL-33 and HMGB1 alarmins promote pro-inflammatory gene transcription via NFκB, a major inflammatory pathway activation(Cayrol and Girard 2014; Cohen et al. 2010; Yang et al. 2015). Tsugita *et al.* group recently identified- SR-B1 as a specific

binding receptor for amorphous and crystalline nanosilica. SR-B1 facilitates silica internalization, followed by canonical NLRP3 inflammasome activation and IL-1β release in both human and mouse macrophages(Tsugita et al. 2017b) (Figure 2). At the same time, Nishijima *et al.* reported human macrophage scavenger receptor A1 (SRA1/MSR1) mediated amorphous silica-induced inflammation is ligand size (50nm) specific. Both particulate endocytosis and receptor-mediated pro-inflammatory signaling occurs in response to silica exposure(Nishijima et al. 2017). Amorphous SiNPs significantly enhances TNF-α and impedes IL-6 production in macrophages stimulated with lipopolysaccharide. 50 nm SiNPs were the most cytotoxic and phagocytosis-independent. Interestingly, amine surface-modification reduced SiNPs-induced dose-dependent cytotoxicity in RAW264.7 macrophages(Uemura et al. 2016).

IL-1α is a constitutively and ubiquitously expressed cytokine(Dinarello 2011), frequently released in epithelial cells(Berda-Haddad et al. 2011; Suwara et al. 2014) or macrophages(Lamacchia et al. 2013; Rider et al. 2011; Sarih et al. 1993). Researchers identified C57BL/6 mice (lung extracted) macrophages as a major source of available IL-1α and driving significant IL-1α release in response to nanosilica(Rabolli et al. 2014). Resident macrophages have already been implicated in IL-1α release under inflammatory conditions in the lungs and brain(Botelho et al. 2011; Luheshi et al. 2009). Amorphous nanosilica induces strong IL-1α secretion and inflammation than crystalline silica particles. Data shows rapid release of constitutively available IL-1α in the alveolar region upon silica exposure, even before IL-1β induction and pulmonary neutrophil influx. Thus, IL-1α directly induces pro-IL-1β production by alveolar macrophages, such that IL-1α neutralization impairs mature IL-1β release and inflammation in silica-treated mice. Endogenous IL-1α release emerged as an early and crucial event for stimulating pro-IL-1β production in SiNPs treated macrophages. Further evidence

suggest IL-33 release in the lung upon silica instillation(Rabolli et al. 2014). TNF-α release is rapid and independent of transcriptional induction. TNF-α is capable of inducing NF-kB activation, pro-IL-1β production and subsequent IL-1β release(Hillegass et al. 2013).

2.4 Nano-Bio Interface: Nanosilica Characteristics and Interacting Biological Systems

The relationship between nanosilica, immunotoxicity and physicochemical properties such as size or surface characteristics are not fully understood. There has been increased debate related to human health and safety aspects regarding the use of engineered nanoparticles in consumer products. The unique protein corona on nanoparticles is a key factor at the nanobio interface, that determines biological reactivity and fate of nanoparticle *in vivo*. Corona composition is often determined by the biological molecules encountered during the entry and on the route of exposure to the nanoparticle (Figure 1). The commercial labels often lack information regarding the morphology, surface characteristics and the concentration of nanoparticles added to the product. Athinarayanan *et al.*, experimentally determined the silica content to be 2.74–14.45μg/g in commercial food products using inductively coupled plasma optical emission spectrometry. The presence of approximately 10–50 nm sized silica nanoparticles (amorphous; spherical) in food was confirmed. Further analysis revealed dose-dependent cytotoxicity, altered ROS levels, gene expression and cell cycle progression in SiNPs exposed human lung normal fibroblasts (WI-38)(Athinarayanan et al. 2014).

The production method of amorphous silica also affects the nanotoxicity, pyrogenic SiNPs are more biologically reactive than precipitated SiNPs. Pyrogenic SiNPs, when incubated *in vitro* absorb more serum proteins on their surface, causing an increased oxidative stress and ROS production. The inflammatory responses were evaluated with *Nos2* induction, NO production, TNF-α, IL-6 and IL-1β secretion in murine macrophages(Di Cristo et al. 2015). The findings

indicate how similar nanomaterials generated through different production methods are not biologically equivalent. Dispersion medium also modulates SiNPs induced biological effects and particle agglomeration causing a change in actual surface area of NPs instilled into the target tissue. Brown *et al.*, found 50nm SiNPs cause more inflammation than 200nm SiNPs in rat lungs. Mice treated with plain and aminated 50nm SiNPs (NPs dispersed in saline) had increased nuclear colocalization of Nrf2, transcription factor. However, results for 50nm and 200nm aminated particles dispersed in bovine serum albumin (BSA) or lung lining fluid (LLF) were not significantly different(Brown et al. 2014). Similarly, corona formed in plasma did not evoke any significant inflammatory response. However, bronchoalveolar fluid (BALF) caused two-fold increase in diesel exhaust NPs uptake and IL-6 release in macrophages(Shaw et al. 2016). Differential proteomics reveal amorphous silica-induced molecular responses in macrophages (RAW264.7) and plasmacytoma cells (MPC11). Several proteins related to AMP-activated protein kinase (AMPK) and myeloid-differentiation primary response protein 88 (MyD88) pathways were modulated upon SiNPs (37nm) treatment in macrophages(Dalzon et al. 2017). In another study, amorphous SiNPs (18nm, 54nm) enhanced monocyte-endothelial cell adhesion by inducing ICAM-1 and VCAM-1, adhesion molecules expression. Interestingly, amorphous SiNPs induced adhesion effect in human endothelial cells was particle size and co-cultured cell type-dependent(Napierska et al. 2013).

Research efforts are being made to evaluate the effect of nanosilica surface chemistry on biocompatibility. The diameter of nanoparticles plays an important role in particle-cell interactions (Table 1). The cytotoxicity behavior of SiNPs is independent of their surface charge in human glioblastoma cells (U373MG)(Kim et al. 2014). 20 nm SiNPs are more toxic than the 100 nm NPs and ZnO NPs are more toxic than SiNPs. Interestingly, both SiO_2 and ZnO

nanoparticles induce apoptosis by caspase-3 activation and DNA fragmentation(Kim et al. 2014). Silica NPs surface area correlates with the size at lower concentrations, as 12nm silica causes high cytotoxicity(Decan et al. 2016). The 12 nm amorphous SiNPs emerge as most potent nanoparticle with significant positive correlation between particle surface acidity and biological potency including cytotoxic and inflammatory responses across the cell lines(Breznan et al. 2017). The surface modification of amorphous nanosilica alters the biological impact of these nanoparticles. *In vivo* mice experiments confirmed, bare SiNPs (0.5 mg) elicit significantly high inflammatory response with increased neutrophil infiltration in bronchoalveolar lavage (BAL) fluid as compared to amine-functionalized SiNPs. These findings suggest surface functionalization as an important factor for reducing SiNPs-induced lung inflammation and improved overall biocompatibility of the nanomaterial.

2.5 Conclusions and Future Directions

Amorphous nanosilica is used ubiquitously across the manufacturing, food, healthcare and biomedical industry. The exponential growth of nano-based applications has increased human nanosilica exposure in both occupational and environmental settings. Several industries use engineered nanosilica particles to improve the quality of their products. Amorphous nanosilica is used extensively across the construction industry to modify concrete microstructure for increased compressive strength and enhanced durability, with reduced pore-size distribution and water sorptivity (Du et al. 2014) (Figure 1). Impregnated amorphous SiO2 in highly ordered conducting mesoporous carbon composites (SiO2-MC) provides high electrochemical activity, reversible specific capacity and cycling performance of the composite (anode) in lithium ion batteries(Hao et al. 2016). These lithium batteries are both single-use and rechargeable with a wide-range of electronics and biomedical applications. Similarly, improper nuclear waste

treatment and stabilization exerts longstanding hazardous impacts on the environment. Incorporation of naturally occurring uranium (U) into amorphous silica (a stable U-Si complex), presenting a promising strategy for an effective and stable remediation of uranium(Massey et al. 2014). Amorphous SiO2 hydrogels, nanostructured materials with high specific surface area might serve as a promising host for impregnating nuclear waste(de Souza et al. 2017). Amorphous silica thin films have been utilized for enhancement of solar photovoltaics to reduce the usage of semiconductor material. Researchers have achieved plasmon-enhanced photoconductivity in amorphous silicon thin films by using thermally stable silica-coated gold nanorods(Chang and Rothberg 2015). Thus, amorphous nanosilica has tremendous engineering and biomedical applications.

However, recent research articles have shown amorphous silica nanoparticles as a potent inducer of inflammation, cytotoxicity and altered immunological responses in humans (Figure 2). The unintended splurge of reactions, nano-bio interactions and extent of damage caused is dependent upon attributes, including particle characteristics- particle concentration and exposure time, mode of exposure, cell type and strong specificity towards the biological system (Table 1). High stability and low biodegradable behavior adds to the strong possibility of amorphous silica induced long term harmful effects on human health, especially in individuals with infections and immunocompromised diseases. Nanosilica entry into the system initiates a wide array of inflammatory signaling cascade leading to disrupted immune signaling and homeostasis. Above findings highlight nanosilica induced biological and cytotoxicity effects, raising concerns over human health and environmental safety (Athinarayanan et al. 2014; Nabeshi et al. 2011b; Shirasuna et al. 2015; Tarantini et al. 2015; Yu et al. 2016). It is essential to understand nanosilica cellular uptake mechanisms and immune cell interactions for identifying the human

population at risk. The adverse effects should be minimized by monitoring the use of nanosilica in consumer-related products, such as mandated proper labeling of nanomaterials used in food additives. Despite the predominant and astounding applications, amorphous nanosilica-induced inflammation, cytotoxicity, immunomodulatory changes and long term effects on human health are the major limitations.

Our review highlights mechanisms underlying amorphous nanosilica induced cytotoxicity, biological and, genetic or epigenetic alterations in humans. Amorphous silica initiated nano-bio interactions may disrupt recognition of future foreign antigen encounters, exacerbate or lead to allergic manifestations, altered adaptive immune responses and other unforeseen adverse health effects. The potential health effects, toxicological and environmental aspects of amorphous nanosilica emerge as major driving forces for the nanotoxicological research and safety assessment, and an offset to amorphous silica-based applications. By understanding the biological basis of amorphous nanosilica induced harmful effects, controlled use of nanosilica-based applications and minimized human exposure modes, the possible adverse effects on humans could be mitigated.

Declaration of interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Captions

Figure 1: Nanosilica characterization, applications and modes of exposure.

Figure 2: Putative cellular mechanisms of amorphous nanosilica mediated inflammation and toxicity.

figure 1

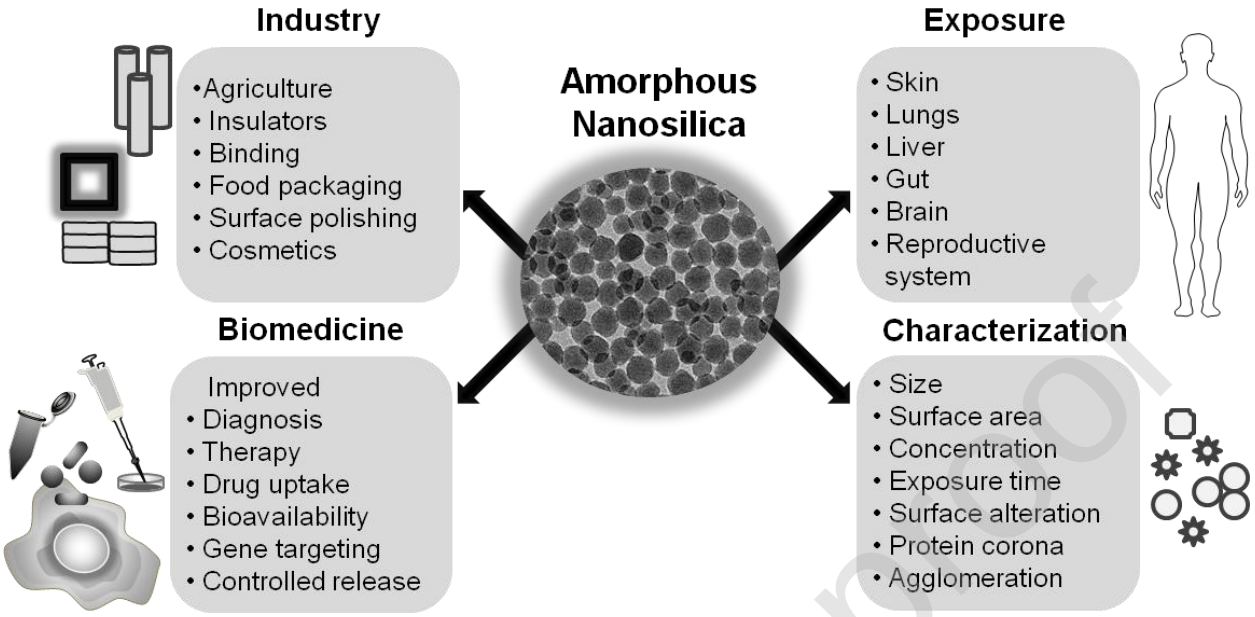
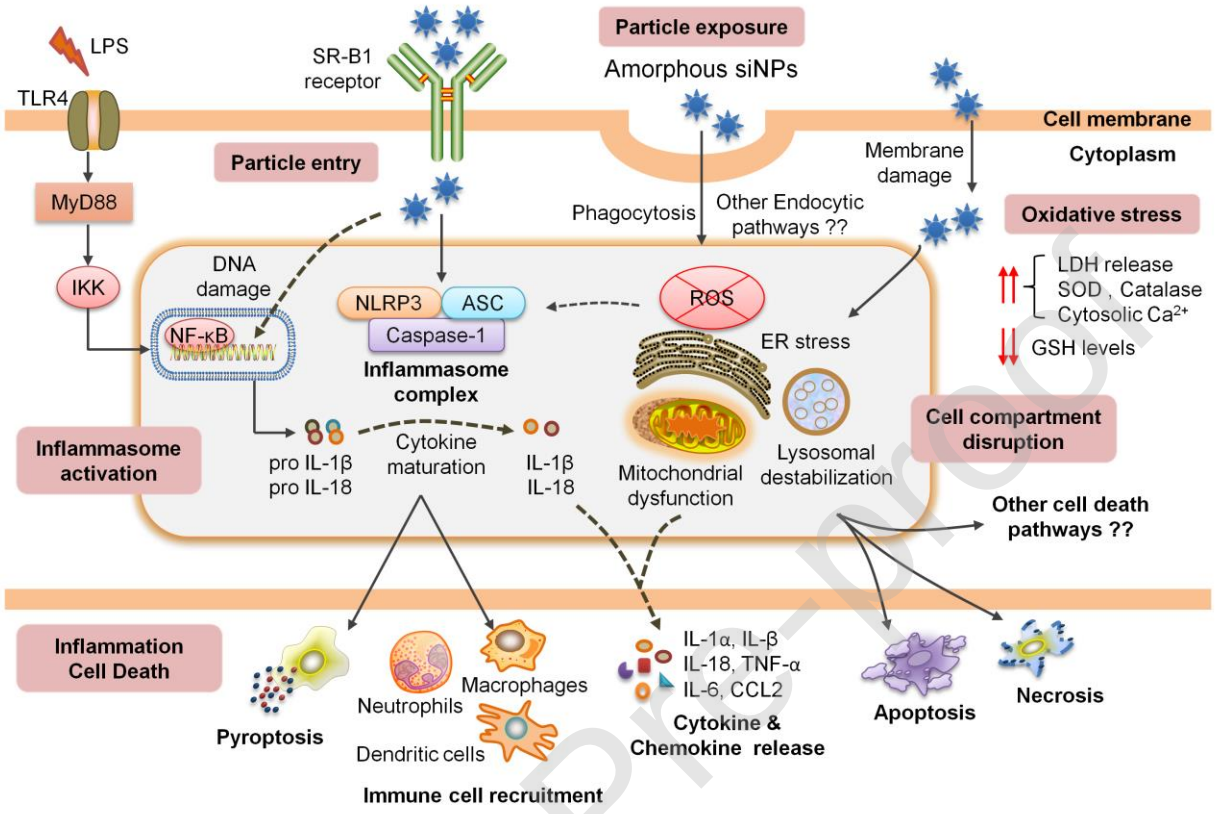


figure 2



Amorphous SiNPs	Concentration	Exposure Time	Study Models	Mechanism of action	References
Fumed silica, 14nm	20-50 μg/cm^2	18 hours	Bone marrow derived dendritic cells (BMDCs) from WT, *Nlrp3*$^{-/-}$ and *Casp1*$^{-/-}$ mice	- Particle uptake partly mediated via actin-mediated endocytosis - ↑ NLRP3, CASP-1 and IL-1β activation	(Winter et al. 2011)
15, 20 and 12 nm	3, 10, 30 and 100 μg/cm^2		Human lung epithelial (A549), human monocytes (THP-1) and mouse macrophage (J774A.1) cell lines	- J774A.1 most sensitive to SiNPs - 12nm: most cytotoxic - Differential cellular cytokine /chemokine release - 12nm: ↑ IL-1β, IL-6 and TNF	(Breznan et al. 2017)
10,25,50 and 100 nm	1, 5, 25 mg/mL	24 hours	human umbilical vein endothelial cells (HUVECs)	- Cytotoxicity; Dose-effect relationship; Negative size-effect - Apoptosis, Caspase-9,7,3 activation - ↑ p53 and Bax, ↓Bcl-2	(Wang et al. 2018)
12, 5–10 and 10–15 nm	12.5, 25, 50, 100 μg/ml	24 hours	Mouse lung epithelial (FE1) cells derived from Muta™ mouse	- 12nm: most cytotoxic - Oxidative stress, Micronucleus formation - 12 nm and LAMP1 highly co-localize - 12nm: affects endosomal, lysosomal and vacuole related pathways	(Decan et al. 2016)
43.11 ± 5.89 nm, monodisperse, near-spherical	SiNP groups (7, 21, 35 mg/kg)		80 female Balb/c mice; SiNPs - tracheal perfusion every 3 days; five times in 15 days	- Oxidative stress - Apoptosis, ↑Caspase-9,3 - ↓ Bcl-2/Bax ratio - Reproductive hormonal imbalance - Reversible follicular damage	(Liu et al. 2018)
12 nm Stober silica, amorphous	2,5 mg/mouse	3 hours	Female C57BL/6 mice, BALF ; mouse macrophage (J774A.1)	- Toxic:12nm Si > crystalline si - IL-1α release precedes IL-1β production and neutrophils accumulation - 12nm: ↑ IL-1α release *in vitro*/ *in vivo*	(Rabolli et al. 2014)

64 and 46 nm, spherical, monodisperse	10,20,50, 100 µg/ml	24 hours	Human hepatic cells (L-02)	- 46nm: most toxic; ↑cellular uptake - Membrane and oxidative damage - Subcellular localization - cytoplasm, lysosomes, vacuole, mitochondria	(Li et al. 2016)
30,70, 100 nm -amorphous; 300,1000 nm micro silica	500µg/mouse	7 days	Balb/c mice (female, 6–8 weeks); intranasal SiNPs. Nasal cavity, lung and liver isolated after 24 hrs	- SiNPs localize into nasal cavity, lung and liver hepatocytes - Activation of coagulation factor XII and platelets	(Yoshida et al. 2013)
Fluorescent 25 and 41 nm, amorphous, spherical	50µg/ml	24 hours	Human monocyte-derived macrophages, Human TAM and alveolar macrophages, THP1 cells	- 25nm: most toxic; ↑cellular uptake in primary human TAM and M2-polarized THP-1 macrophages	(Hoppstädter et al. 2015)
Rhodamine labeled 25,50 nm, amorphous, pseudo-spherical	100µg/ml	2- 24 hours for cell line; 2,30-360 min. for tissue	Human choriocarcinoma (BeWo) and perfused placental tissue	- Cytotoxic effects - SiNPs accumulation in placental tissue	(Poulsen et al. 2015)
FITC labeled 70 nm, unlabeled 70nm	0.04 and 0.025 mg/g body weight of siNPs in mice 10-100µg/ml for human placental cells	2-6 hours	C57BL/6J (wild-type) and Slc:ICR mice; Mouse primary placental cells and human placental cells (Sw.71)	- ↓Body weight/ fetal weight - Neutrophils and macrophages infiltration in placenta - ↑ IL-1β, TNF-a, IL-6 and CCL2 - ↑caspase-1 activation - Body weight significantly restored in pregnant *Nlrp3*$^{-/-}$ mice - ↑ IL-1α release in *Asc*$^{-/-}$ mice	(Shirasuna et al. 2015)
Fluorescent labeled 30, 40, 50 nm, stober, amorphous, monodisperse	20 cm^2 SA (EA.hy926) and 10 cm^2 SA (hPAEC) per cm^2	12 hours	Human endothelial cell line (EA.hy926) , primary human pulmonary artery endothelial cells	-↑ICAM-1 and VCAM-1 expression -↑ monocytes-endothelial adhesion - cytoplasmic localization of SiNPs	(Napierska et al. 2013)
Rhodamine-labeled and	Mice [5mg/kg (~100µg/head)]	3-6 hours	C57BL/6 N female mice (6–7 weeks old), primary	-↑IL-1β secretion from BMDMs - SiNPs localize within lysosomes	(Tsugita et al. 2017a)

unlabeled 30nm, amorphous, modified stober	BMDMs - 100 μg/cm^3		mice BMDMs		
10-20nm, amorphous powder	100-500μg/ml	1,6,18,24,48 hours	human healthy lung epithelial cells(L-132)	- Cell death - ↑ LDH levels, ↓ GSH levels - IL-8/COX-2 ↑ and Mitochondria ↓ with increased SiNPs exposure	(Sahu et al. 2015)
30 nm, amorphous,	FITC/ Rhodamine-labeled SiNPs (1-3μg/mL)	30 minutes	Scarb1$^{+/-}$ and C57BL/6 mice, mouse BMDMs and human PBMCs, NIH 3T3 and HEK293 cells	- SR-B1 is a tethering receptor for silica - ↑ IL-1β and IL-1α release - canonical inflammasome activation	(Tsugita et al. 2017b)
10, 20, 40, 60, 80, 100,150 and 200 nm, amorphous, monodisperse	10-500 μg/ml	For 24, 72 hours	Human lung epithelial (A549), human hepatoma (HepG2), mouse embryonic fibroblast (NIH/3T3)	- cytotoxic, ↑ROS levels - 100/200nm: ↑ROS production - 60nm: ↑cellular uptake	(Kim et al. 2015)
30, 50, 70, 100, 300, or 1,000 nm, amorphous	12.5-200 μg/ml	For 6, 12, or 24 hours	Human THP1 monocytic cell line	- Inflammation: Particle size dependent - 50nm: ↑IL-1βand TNF-α release	(Nishijima et al. 2017)
10–20 nm, spherical		30, 100, or 300 μ g/mL, for 3 or 6 hours	HEK293 cells, Primary BMDMs from C57BL/6 male mice	- Differential cytotoxicity - Disrupted Ca^{+2} homeostasis - TRPM2 Ca^{+2} channel regulates NADPH oxidase and ROS production upon SiNPs exposure	(Yu et al. 2015)

Table 1: Toxic effects of amorphous nanosilica and altered biological functions.