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Genotoxicity of Amorphous Silica Nanoparticles: Status and Prospects

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Abstract

Amorphous silica nanoparticles (SNPs) are widely used in biomedical applications and consumer products. Little is known, however, about their genotoxicity and potential to induce gene expression regulation. Despite recent efforts to study the underlying mechanisms of genotoxicity of SNPs, inconsistent results create a challenge. A variety of factors determine particle-cell interactions and underlying mechanisms. Further, high-throughput studies are required to carefully assess the impact of silica nanoparticle physicochemical properties on induction of genotoxic response in different cell lines and animal models. In this article, we review the strategies available for evaluation of genotoxicity of nanoparticles (NPs), survey current status of silica nanoparticle gene alteration and genotoxicity, discuss particle-mediated inflammation as a contributing factor to genotoxicity, identify existing gaps and suggest future directions for this research.

Keywords

Silica nanoparticles; Genotoxicity; Gene expression profile; Inflammation; Structure-activity relationship

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1. Introduction

Currently, there are over 1,800 commercially available nanotechnology-based products listed in the Consumer Products Inventory (CPI) from 622 companies.[1] According to CPI, nanomaterial components have been classified into five major categories of silicon, carbonaceous, metal, “not advertised,” and “other”. Over 100 commercial products contain synthetic silica nanoparticles.[1] The roles of silica nanoparticles in these products include anti-caking agents in the food industry, anti-foaming agents, and viscosity controlling ingredients in the beverage industry, excipients in drugs and vitamins,[2—4] and components of artificial implants due to osteogenic properties.[4–6] Silica nanoparticles have also been used as viscosity stabilizers, as well as bulking, abrasive, opacifying, absorbent, and suspending agents in cosmetic products.[7–9]

Despite their extensive use, little is known about the genotoxicity of silica nanoparticles (SNPs). While amorphous silica does not introduce similar oral, skin, or inhalation risks as crystalline silica, which has already been extensively reviewed in the literature,[10] potential concerns regarding the safety of amorphous silica have not been critically examined. The human body can be exposed to silica nanoparticles by intentional or unintentional exposure through several routes such as inhalation, oral ingestion, parenteral injection and transdermal penetration Exposure to SNPs could potentially have adverse effects on human health through direct or indirect mechanisms.[11,12] International Agency for Research on Cancer (IARC) has classified crystalline silica as group 1 carcinogenic compound to humans. However, the carcinogenicity of synthetic amorphous silica has not been recognized yet by IARC because of insufficient evidence. It is noteworthy that for amorphous SNP classification, the role of size has not been considered. The concern of risk-to-benefit ratio of widely used nanometric amorphous silica particles remains valid, due to lack of established data on genotoxicity and limited information on the impact of their physicochemical properties on safety and health risk assessments.[13] In contrast to relatively well-documented cytotoxicity studies of amorphous SNPs, there is a substantial gap in our understanding of the genotoxicity of silica nanoparticles as a function of heir physicochemical properties. Systematic cytotoxicity and genotoxicity studies, therefore, are necessary to understand the safety and environmental impact of amorphous silica nanoparticles.[14] The vast majority of studies to-date have assessed either the direct cytotoxicity or inflammatory responses of SNPs. It has been shown that although cells exposed to sub-toxic concentrations of SNPs may not demonstrate significant viability or phenotypic changes, altered regulation of thousands of genes occurs.[15] While immediately noticeable toxicity may not appear in such cases, induced altered gene expression could potentially impact the cell’s capability to react to stressors.[16] Reported studies shed light on acute cytotoxic effects. Therefore, a detailed investigation to address global gene expression, regulation, and the underlying mechanisms in response to SNPs is required.

Herein, we provide a literature review to identify methodologies and strategies commonly used to assess genotoxicity; summarize achievements and limitations of available studies investigating genotoxicity and alterations in the gene expression profile as a result of exposure to silica nanoparticles (SNPs); discuss inflammation and its direct and indirect

effects on genotoxicity and alterations in the gene expression profiles commonly associated with SNPs; highlight current gaps and provide suggestions on how to fill them.

2. Influence of physicochemical properties on toxicity of SNP

The influence of physicochemical properties of SNPs such as size, surface charge, geometry, and porosity on their toxicity has been investigated in a number of studies. These studies suggest correlations between physicochemical properties on one hand and cytotoxicity, cellular internalization, and co-localization on the other, in various cell lines exposed to SNPs.[17–22] For example, size-dependent cytotoxicity of SNPs in human endothelial cells[23] and macrophages[24] has been reported. Protein corona formation on SNP coats the accessible silanol groups on the particle surface thereby modifying the surface charge and reducing the toxicity compared to SNPs tested under serum-free culture conditions.[17, 25] Similar nonspecific protein adsorption profile is observed for 50 and 500 nm nonporous SNPs indicating size independent protein corona profile formation on particles. However, it has been shown that size influences the mechanism of uptake by murine alveolar macrophage (RAW264.7) cell lines.[17] Plain SNPs ranging in size from 50 nm to 500 nm and their NH2- and COOH-functionalized counterparts were added to colorectal carcinoma (HCT116), and human prostate carcinoma (DU145) epithelial cell lines, and Raw macrophages (RAW264.7). Cell type- and concentration dependent uptake and toxicity were observed, with RAW264.7 cells being more susceptible to uptake and toxicity compared to epithelial cells. Enlargement of RAW264.7 macrophages was observed compared to untreated cells.[24] In other studies, an increase in toxicity and oxidative stress with smaller SNPs (size ranges from 20 nm to 500 nm) was observed in human hepatoma HepG2 cells and neuronal GT1–7 cells.[26] Surface modification is another factor that influences SNP toxicity. Thiol or amino-modified SNPs compared to unmodified ones showed reduced toxicity in human neuroblastoma cells.[27] Cell type- and concentration-dependent toxicity of SNPs was also confirmed in other studies aiming to investigate the impact of porosity, geometry, and surface functionalization on cellular toxicity by nonporous and mesoporous SNPs.[28] The cellular association, plasma membrane damage, and cytotoxicity of SNPs in macrophages were directly related to their porosity and surface charge.[28] However, the same correlation between these biological properties and particle geometry was not confirmed.[28,29] Higher silanol density of nonporous particles resulted in higher cellular association compared to their mesoporous or surface modified counterparts.[28] Similarly, another study demonstrated that changing geometry of SNPs from spherical to cylindrical or worm-like particles with the same size (~200 nm in diameter) does not govern cell association, toxicity, or membrane integrity.[30] Interestingly, geometry played an essential role in determining the mechanism of uptake in that at early time points spherical particles were internalized primarily by clathrin-mediated endocytosis, while high aspect ratio particles were taken up by the cells primarily by phagocytosis or macropinocytosis.[31] These particles showed a concentration threshold (~100 μg/mL) for uptake, with limited to no impact on membrane damage, mitochondrial function, or cell death below this threshold regardless of their geometry.[30]

In addition to size, other parameters such as aspect ratio, porosity, and surface modifications were found to influence nanoparticle biodistribution.[20,32–34] SNPs primarily accumulate in the organs of the mononuclear phagocytic system such as liver and spleen. Greater

accumulation in the lung has been observed with an increase in nanoparticle size, porosity, and aspect ratio.[32] While porosity and increased aspect ratio led to accumulation in the lung, amine modification of SNPs decreased lung accumulation.[32] Increased porosity resulted in increased hydrodynamic size in the presence of serum. Larger hydrodynamic size leads to mechanical obstruction in vessels leading to congestion in several organs compared to nonporous SNPs.[32] A study investigating maximum tolerated dose (MTD) of nonporous and mesoporous SNPs with aspect ratios of 1, 2, 4, and 8 and approximately 120 nm in diameter demonstrated that mesoporous SNPs exhibited lower MTD and, therefore, are more toxic than nonporous SNPs.[20] Figure 1 summarizes the trends observed in the *in vitro* and *in vivo* toxicity studies of various SNPs. Together these studies suggest the clear impact of physicochemical properties of SNPs on toxicity. However, the underlying mechanisms and pathways of these toxicities are not fully understood. Below we review studies which explain some of these effects and warrant further mechanistic investigations.

3. Genotoxicity of SNPs

Literature survey for toxicity and genotoxicity of NPs in the Scopus database using keywords “NPs and toxicity,” “NPs and genotoxicity,” “SNPs and genotoxicity and comet assay,” and “SNPs and genotoxicity and micronucleus assay” have been conducted. The results demonstrated a total of 18,858 papers published on NP toxicity. Among these publications, 1,265 papers were related to SNPs. A total of 1,308 articles were published on the subject of NPs and genotoxicity. Among them, only 106 publications described experimental studies on SNPs genotoxicity. Thirty-five (35) and 26 out of these 106 published studies evaluated genotoxicity of SNPs using comet assay and micronucleus assay, respectively. Titanium dioxide, silver, zinc oxide, and iron oxide NPs are among the best-studied materials in the area of genetic toxicology. There is a considerable recent rise in the investigation of genotoxicity of SNPs as evident by the increase in the number of studies conducted since 2006 and over 80% of the studies conducted since 2013 (Figure 2).

Genotoxic impact of SNPs in cells can be defined as primary or secondary effects or classified as direct or indirect effects.[35] Nanogenotoxicology by definition is the ability of nanomaterials to induce DNA mutations, abnormal gene expression, or chromosomal aberrations such as chromosomal breaks, fragmentation, chromatid and chromosomal gaps, and chromatid deletions.[36, 37] DNA damage such as crosslinking, single-stranded breaks, double-stranded breaks, or impairment in DNA repair has been reported.[38] Inconsistent results were observed for genotoxicity of silica nanoparticles. For instance, comet assay of colloidal amorphous spherical 20.32±2.23nm and 30.51±3.31nm (measured by TEM) SNPs obtained from Sigma (Ludox CL 420883 and Ludox CL 420891) on 3T3-L1 fibroblast cells which were exposed to 4 or 40 μg/ml for 3, 6, and 24 hours showed no significant genotoxicity.[39] Similarly, mouse fibroblasts exposed to 15–100nm SNPs for up to 72 hours at 1–100 μg/mL did not show genotoxic or cell morphological transformations despite internalization.[3] However, in another study, SNPs with almost the same size (34nm) induced gene mutations and chromosomal aberrations in 3T3-L1 mouse fibroblasts and mouse embryonic fibroblasts (lacZ reporter gene) at non-cytotoxic 4 μg/ml or 40 μg/ml single treatment for 24 hours.[5]

There is also a scarcity of reliable studies evaluating the influence of geometry, porosity, density, surface charge, and surface roughness on induction of genotoxicity. Therefore, despite recent reports, the overall picture of SNP genotoxicity remains incomplete. Therefore, controversial issues regarding reported cytotoxicity vs. genotoxicity of nanomaterials is worth a special attention.

Drawing general conclusions without elucidating the exact conditions under which experiments were conducted is a common pitfall in the majority of the published studies. The controversy, therefore, could be overcome by identifying the experimental design and conditions used to analyze the SNPs. Important experimental factors include particle physicochemical properties, concentrations studied, temperature ranges, treatment volumes, cell culture conditions, and the protocols utilized. We review *in vitro* and *in vivo* studies of SNP genotoxicity and analyze data in the context of methodologies which were used to assess genotoxicity and gene expression alterations.

3.1. In vitro studies

At high cytotoxic doses, some silica nanoparticles induce the formation of micronuclei. At non-cytotoxic doses, no such activity is observed.[40–43] These observations are consistent between different cell lines (V79 vs. A549 vs. 3T3-L1 fibroblasts) and different silica nanoparticles (silica gel vs. synthetic amorphous silica nanoparticles produced by Stober method).[40–43]

Ludox® (Sigma Aldrich) colloidal silica nanoparticles' genotoxicity was assessed on MCF-7 human breast cancer cell lines at 4 mg/L and 40 mg/L by the comet assay. A positive genotoxic response of particles was detected by the statistically significant difference of the mean Olive tail moments (OTM) of SNPs and negative control. The level of DNA damage by SNPs was comparable with 1 μM B[a]P.[44] However, in a similar study, genotoxicity assessment of different size alumina-coated SNPs utilizing the comet assay in 3T3-L1 mouse fibroblasts showed no concentration-, time- or particle size-dependent genotoxicity.[39]

Generalization in nanotoxicity studies creates a barrier preventing accurate comparison between studies conducted in different laboratories and halt harmonization of research approaches to study SNPs. For example, one study conducted in mouse 3T3 fibroblasts[3] concluded that SNPs are neither cytotoxic nor genotoxic. However, a closer look at the experimental design reveals that the cytotoxicity experiments were conducted at concentrations not exceeding 100 μg/mL, while genotoxicity findings were only supported by the micronucleus test and did not include other methods, such as comet assay or gene sequencing, to verify the original findings. In contrast, another study employing the comet assay and conducted in human peripheral blood lymphocytes and human embryonic kidney (HEK293) cells indeed revealed genotoxic effects at the particle concentration of 100 ug/mL. In this case Ludox® colloidal silica nanoparticles with a nominal diameter of 6, 15, 30 and 55 nm were used to further extend the investigations.[45] The micronucleus test was performed in 3T3-L1 mouse fibroblasts treated with four different sizes of amorphous SNPs: 11nm (ξ −43.3 mV), 34nm (ξ −33.7 mV), 34nm (ξ −10.6 mV), and 248nm (ξ −49.1 mV). The results illustrate chromosomal aberrations in response to only 34nm particles.[5] Another study employing comet assay in the same cell line and the same particles did not show any

genotoxicity at sub-toxic doses of 4 and 40 μg/ml.[39] The absence of genotoxic response to 11nm and 248nm particles in this study was attributed to hydrodynamic size. Interestingly, particles with the nominal size of 11nm formed agglomerations with a hydrodynamic diameter of 103.1nm. Therefore these agglomerates and particles with the hydrodynamic size of 248 nm were larger than the 34 nm particles, which did agglomerate. The large size of 248 nm particles and agglomeration of 11 nm particles, decreased the surface area available for the interaction with cells as compared to 34 nm particles at the same mass concentrations.

Genotoxicity of SNPs was tested using the comet assay on three epithelial cell lines of HaCat, HT29, and A549 exposed to amorphous SNPs with a nominal diameter of 14 nm at concentrations up to 10 μg/ml. In cell culture medium these particles formed agglomerates with hydrodynamic diameters of 500 nm, disrupted cell membrane, dispersed inside the cytoplasm, and showed significant DNA damage.[46] In this study SNPs were dosed in serum-free culture medium which explains the reason for the agglomeration of particles, increased interaction with cell membrane, and elevated cyto- and genotoxicity. However, for the same cell line (A549) SNPs with diameter of 50 ± 3 nm at concentrations up to 100 ug/mL did not show any significant increase in DNA repair activity or DNA damage. This study was conducted in the culture medium supplemented with serum. The results of this investigation suggest the inability of SNPs to induce genotoxicity, likely due to the neutralization of the cell reactive moieties on the particle surface by serum proteins spontaneously adsorbed on the particle surface.[47] In agreement with this data is another study evaluating the influence of size, charge, and protein adsorption of amine-terminated and non-amine-terminated SNPs with diameter of 50 and 200 nm. This study demonstrated that bovine serum albumin (BSA) and serum adsorption on particle surface decreased genotoxic effects compared to particles dispersed in Hanks' balanced salt solution (HBSS) or lung lining fluid (LLF). Decreased genotoxicity of the protein-coated particles detected by the comet assay was attributed to the change in surface reactivity, decreased cellular uptake, and presence of antioxidant in serum and BSA. In addition, smaller (50nm) SNPs showed pronounced DNA damage compared to their larger (200nm) counterparts. In the case of 50nm particles the study reported oxidative damage even at the lowest tested concentration of 31.25 μg/ml.[48] An increase in the particle zeta potential after protein adsorption, and some degree of agglomeration was seen in different media evaluated in this study.[48] The authors attributed genotoxicity to the alteration in the particle zeta potential. However, closer look at the data reveals that particle zeta potential in different media was almost the same.[48] Therefore, it is unlikely that zeta potential was the main physicochemical parameter contributing to the particle genotoxicity. Interestingly, the same study did not find genotoxic effects for studied particles when the micronucleus test was used for analysis instead of the comet assay.[48] Different sensitivity of the micronucleus test vs. comet assay is not surprising in this case, because each procedure assesses different genotoxicity endpoints as discussed above.[49] In a recent published report, a DNA repair deficient cell line (chicken DT40) was employed to increase sensitivity of genotoxicity experiments by spherical and rod like mesoporous SNPs at a range of concentrations including 10, 25, 50, 75, and 150 μg/ml. A DNA repair deficient cell line compared to DNA repair proficient cells allowed to observe the induced genetic aberrations by preventing quick DNA repair. In this study, both particles showed less genotoxicity in

wild type cells compared to a DNA repair deficient cell line. Although this study reported that rod shape particles are more genotoxic due to the geometric variation, simultaneous variations in size and shape makes it difficult to attribute the observed dissimilar genotoxic responses to either size or shape effect alone.[50]

Accumulation of SNPs in vacuoles and their absence in nucleus provided by TEM imaging suggests indirect genotoxicity mechanisms of SNPs.[5] The proposed indirect genotoxicity mechanisms is correlated with induction of ROS and consequently oxidative DNA damage. It is postulated that SNP accumulation in cytoplasm vacuoles cause mechanical hindrance of mitotic processes.[5]

3.2 In vivo studies of SNP genotoxicity

There are few *in vivo* genotoxicity studies on SNPs (Table 1).[51] SNPs with a nominal size of 15 and 55 nm and gold nanoparticles with size of 2, 20, and 200 nm were administered to male Wistar rats by three consecutive intravenous injections at different doses (25–125 mg/kg) and at time intervals (48, 24, and 4 hours) prior to tissue collection. Genotoxicity for both 15 and 55 nm SNPs was observed at their MTD (50 mg/kg vs. 125 mg/kg, respectively) by comet and micronucleus assays. The authors suggest an indirect secondary genotoxicity mechanism driven by the inflammatory response induced by these particles.[51]

In addition to the physicochemical properties of particles, other study design parameters, including but not limited to, the dose, exposure time, and the route of exposure are also important and may influence the study outcome. For example, Young Kwon et, al,[52] studied genotoxicity of 33 and 90 nm SNPs at a range of concentration using both *in vitro* and *in vivo* methods. The particles were administered to male Crl: CD SD rats by oral gavage three times at 0, 24, and 45 hours and the genotoxicity was assessed by the comet assay.[52] In the same study particles were administered by the same route to ICR mice with a 24-hour interval, and genotoxicity was assessed by the micronucleus test.[52] Both *in vitro* and *in vivo* data obtained in this study demonstrated that SNPs at 175–1,400 μg/mL or 500 to 2,000 mg/kg body weight did not induce statistically significant genotoxicity.[52] Likewise, another study investigating genotoxicity of synthetic amorphous SNPs also demonstrated no significant genotoxic effects after intravenous injection and intratracheal instillation.[13] In this study, rats were exposed to SNPs with a nominal size of 20 nm by intratracheal instillations at three different doses of 3, 6, or 12 mg/kg at time intervals of 48, 24, and 3 hours before tissue collection. Both comet assay and the micronucleus test did not reveal significant DNA damage to the exposed lung and broncheoalveolar lavage fluid (BALF) cells, as well as cells of secondary tissues such as spleen, kidney, blood, bone marrow, and liver.[13] Oral administration of the same particles in male Sprague Dawley rats at 5, 10, or 20 mg/kg/day as well did not induce DNA strand breaks or damage.[34] To ensure the exposure to high dose of the particles, SNPs were also intravenously injected at the same time intervals and at higher doses (5, 10, or 20 mg/kg). Liver discoloration, increased hepatotoxicity blood markers, hepatic histopathological changes, dose-dependent thrombocytopenia, and animal mortality were observed despite the negative genotoxicity findings in the micronucleus test.[13] Comparing the earlier study[51] with the most recent one[13], each study reported almost the same set of experiments of intravenous injections,

with similar sized SNPs (15nm to 20nm), generated contradictory results. However, the administrated doses were different (50 mg/kg vs. 20 mg/kg).

Genotoxicity of spherical nonporous SNPs was studied in rats exposed to the particles by the pulmonary route.[53] In this case particles of two sizes (37 or 83 nm) were tested at two dose levels (3.7×10^7 and 1.8×10^8 particles/cm^3 which is equivalent to mass concentrations of 1.8 or 86 mg/m^3, respectively) and the exposure continued for 1 or 3 days. Micronuclei induction in peripheral blood cells was used as a measure of particle genotoxicity. The rats did not develop any significant pulmonary inflammation and genotoxicity.[53] Protasova et al., reported pathogenic effects of SNPs on early embryo developments. Cyto-, geno-, and embryotoxicity of 12 nm SNPs were assessed in pre-and post-implantation embryos in mice, rats, and human peripheral blood lymphocyte culture treated with these particles at two concentrations (100 and 200 μg/mL). Chromosome aberration assay, micronucleus test, and DNA comet assay in human peripheral blood lymphocytes did not demonstrate chromosome aberrations, micronuclei, or DNA breaks, indicating the absence of genotoxicity at tested concentrations. However, embryotoxicity was detected in murine embryos before the implantation as measured by the inhibited cavitation process and hatching of blastocysts. During the post-implantation stage, brain and caudal artery hemorrhages, brain deformation, brain and pericardial edemas, neural tube dysgraphia, and reduction of cerebral hemispheres were observed at the particle concentration of 200 ug/mL.[54]

Genotoxicity of SNPs was also assessed *in vivo* using alternative models, such as fruit fly. However, conclusions regarding the genotoxicity of SNPs in this model would require further verification.[55]

4. Silica nanoparticle-mediated alterations in gene expression profile

Silica nanoparticles alter gene expression by direct or indirect interactions with different signaling pathways.[38] Impact of SNP's carefully controlled physicochemical properties on pathways leading to alterations in gene expression needs a detailed examination.

4.1 In vitro effects of SNPs on gene expression profile

A hierarchical dose-dependent cascade of events was detected by analysis of the gene expression profile in A549 lung epithelial cells treated with amorphous fumed SNPs (AEROSIL® 200 NPs). The nominal particle size was 10±4 nm and concentrations were 0.1, 1.0, 1.5, 3.0, and 6.0 μg/cm^2. Another important nuance of this study is that the cells were treated in the absence of fetal bovine serum (FBS) which lead to SNP agglomeration up to 350 ± 8 nm. A549 exposed to these SNPs for 24 hours showed 100% viability at doses up to 1.5 μg/cm^2 and differential expression of 255 genes. At higher doses, cell viability decreased while the number of genes with altered expression increased. Specifically, at concentrations of 3.0 and 6.0μg/cm^2 cell viability was 99 and 100%, respectively, and a number of differentially expressed genes were 695 and 2258, respectively. Pathway analysis of differentially expressed genes for each concentration showed concentration-dependent, hierarchical stress response. Specifically, at low concentrations most of the affected genes encoded proteins involved in clathrin-mediated endocytosis and actin cytoskeleton remodeling; mid concentration affected genes encoding proteins contributing to coagulation

and inflammation; and high concentrations resulted in changes in the expression of genes encoding proteins involved in metabolism of xenobiotics (*ALDHA*, *MAP2K3*, and *TNF)* and acute phase response (*NFKB2*, *IL6,* and *SOD2)*.[56] The results of this study[56] are in agreement with the earlier reports of hierarchichical oxidative stress response by Nel et al.,[57] and predictive toxicological paradigm by Meng et. al.[58]

Gene expression alteration and its connection to genotoxicity were evaluated using 100nm negatively charged MSNs in normal human embryonic kidney 293 (HEK293) cells. The analysis was performed by a fluorescent in situ hybridization (FISH) assay and Agilent human mRNA microarray. A set of genes including human telomerase (TERC), epidermal growth factor receptor 2 (HER2), cyclin-dependent kinase inhibitor 2A (CDKN2A), and epidermal growth factor receptor (EGFR) genes were chosen to relate gene expression profile changes to genotoxicity since these genes are routinely used for the diagnosis of certain human cancers. No significant genotoxicity was detected in cells treated overnight with 120 ug/mL of MSNs. However, mRNA microarray assay showed upregulation and downregulation of 579 genes and 1263 genes, respectively. Significant gene expression alteration by MSN in HEK293 cells was accompanied by cell morphological changes, however, no other data on cytotoxicity of MSN particles at tested conditions was reported.[38] The influence on genotoxicity of MSNs in HEK293 cells was also studied by DNA microarray analysis and revealed alteration in the expression of genes involved in chromatin remodeling, chemokine CCL15, and 40S and 60S ribosomal protein homologs.[59]

Focusing on the influence of particle size on altering gene expression, Waters et al.,[60] showed that gene expression regulation induced by different sizes of amorphous silica nanoparticles is dependent primarily on particle surface area rather than particle mass or number. Microarray analysis of treated RAW 264.7 cells with a range of silica nanoparticles from 7 nm to 500 nm in diameter showed overal the same biological trend at similar surface area, regardless of particle diameter.[60]

While *in vitro* assays such as cell viability, uptake, membrane integrity, and apoptosis detection are useful to determine the extent of toxicity of nanoparticles, they have limitations in providing a detailed understanding of the mechanisms of toxicity. A system toxicology approach to elucidate molecular mechanisms is needed to investigate the mechanism of toxicity of nanomaterials. Transcriptomic studies investigating upregulation or downregulation of genes under specific treatments could be successfully used to define molecular alterations leading to toxicity. The emerging Omics science adds to the toolbox of assays for nanotoxicology and is largely unexplored for understanding the underlying mechanisms of nanotoxicity of SNPs.[14,61,62]

4.2. In vivo effects of SNPs on gene expression profile

The expression of stress and toxicity pathway genes were investigated *in vivo* using both traditional (e.g., rodent) and alternative (e.g., zebra fish) models. While rodents are commonly used as a model in toxicology during preclinical development of nanotechnology formulated drugs, zebrafish embryos are widely used in genetics and environmental toxicology. Recently, the utility of this model was expanded to drug discovery due to the relative ease of maintenance, rapid reproduction, sensitivity, cost-effectiveness, and optical

clarity.[63–65] Embryonic and cardiovascular toxicity in zebrafish embryos exposed to SNPs is reported elsewhere.[66]

In vivo studies in rodents demonstrate the robust effect of SNPs on gene expression profiles. For example, in one such study cobalt ferrite magnetic-core nanoparticles (35nm) and silica-coated cobalt ferrite magnetic-fluorescence nanoparticles (50nm) were injected to mice via tail vein, and gene expression alteration profile was analyzed in liver tissue by PCR array including 52 genes.[67] Mouse liver tissue was assessed to identify possible metabolic stress, DNA damage, oxidative stress, growth arrest, heat shock, apoptosis signaling, proliferation, and carcinogenesis, or pro-inflammatory gene up-regulation or down-regulation. Biodistribution studies by whole-body PET imaging showed significant liver accumulation of both particles with 50-fold liver-to-muscle ratio accumulation. Gene expression revealed 2 genes and 22 genes alterations, indicating a significant decrease of stress and toxicity signal pathways gene expression by silica coating of cobalt ferrite magnetic-core nanoparticles.[67]

Genome-wide transcriptional analysis revealed changes in gene expression profile in zebrafish embryos exposed to negatively charged (−38 mV) 62.14 ±7.16 nm SNPs. Gene expression alteration was related to innate immune response, response to oxidative stress, cell signaling, response to stimuli, cellular processes, and embryonic development. Gene signal transduction network analysis showed alteration in 2,515 genes and identified 127 core regulatory genes indicative of particle effects on vascular smooth muscle contraction, gap junction, cytokine-cytokine receptor interaction, apoptosis, and toll-like receptors (TLRs), JAK-STAT, MAPK, and calcium signaling pathways.[68]

5. Understanding the mechanisms of SNP genotoxicity and effects on gene expression profile

5.1. Autophagy and Lysosomal Dysfunction

The majority of known nanoparticle uptake routes converge on the lysosome. Lysosomal overload and rupture play an important role in the production of certain inflammation markers (i.e., IL-1 family cytokines) due to its role in the NLRP3 inflammasome activation, which will be discussed in more detail below. Cells use autophagy to recycle intracellular pathogens, foreign particulate, and aberrant self-proteins. Alterations in lysosome function and autophagy, therefore, affect both cellular homeostasis and responses to foreign substances. As such, autophagy and lysosomal dysfunction are increasingly considered in the context of nanoparticle toxicity. Here, we discuss these pathways in the context of SNP-mediated alteration in gene expression profile.

Both porous and non-porous SNPs were shown to activate the lysosomal pathway. For example, RAW 264.7 macrophages were treated for 4 hours with four types of SNPs to explore the influence of size, porosity, and surface modification with poly(ethylene glycol) (PEG) chains on cell viability.[15] It is known that sub-toxic concentrations trigger cellular defense mechanisms and biological processes that are not noticeable using conventional toxicity assays.[59] Amorphous non-porous spherical SNPs with two different diameters (46

± 4.9 nm and 432 ± 18.7 nm) were compared to mesoporous spherical SNPs of 466 ± 86 nm in diameter and surface modified MSNs. Early gene expression in macrophages treated with sub-cytotoxic concentrations of mesoporous SNPs of various size and porosity, resulted in gene expression modulation without induction of acute toxicity. Non-porous SNPs of similar size did not significantly change gene expression at the equitoxic concentrations. While no significant effects on ROS production, mitochondrial damage, or mitochondrial membrane potential disruption were detected, the lysosomal pathway was activated by the MSNs (Figure 3).[15]

Another study involved microarray analysis and demonstrated that SNPs altered expression of genes encoding proteins critical for lysosomal function as well as those involved in membrane and cytoskeleton integrity.[43] This study investigated the impact of particle size and concentration on the mechanisms of genotoxicity and cytotoxicity. It included the analysis of commercially available silica nanoparticles with a nominal diameter of 12 nm, 5–10 nm, 10–15 nm, and 2 μm.[69] DLS and TEM revealed randomly shaped aggregates of 22.5, 56.9, 237.5, and 2045.4 nm particles, respectively. Cytotoxicity studies performed in the mouse lung epithelial (FE1) cells at a range of concentrations (12.5, 25, 50, 100 μg/mL) revealed both size and concentration-dependent effects.[69] Micronucleus assay confirmed genotoxicity of all nanosized silica particles but not their micron-sized counterparts. The genotoxic effect was observed at the concentration of 12.5 μg/mL and 12 hour time point. The analysis of gene regulation suggested the step-by-step interaction between particles and cells. The particle uptake via endocytosis resulted in membrane and cytoskeleton gene upregulation and accumulation of particles inside the lysosome and potential subsequent clearance.[70–72] Higher lysosomal activation was detected in cells treated with the smallest particles (5–10nm) as compared to SNPs of other sizes and was attributed to increased cellular internalization.[69] The original goal of this study was to analyze 12 nm, 5–10 nm, 10–15 nm, and 2 μm particles, however, due to the low surface charge of particles, large random shape aggregates were formed.[69] Larger aggregates were observed for 5–15 nm nanoparticles. This could potentially decrease uptake due to the difficulty of internalization. Considering the influence of geometry and particle surface orientation in uptake and the subsequent cascade of effects,[31] either preventing aggregation or understanding aggregation states may be essential, if the study is focused on investigating the influence of physicochemical properties such as size. Full characterization of particles is crucial to recognize the genotoxic effect resulting from the specific physicochemical properties.

Several studies attempted to link nanoparticle effects on lipid metabolism and lysosomes in the context of a cell defense strategy against reduced cell viability, maintenance of membrane barrier functions[73], lipid metabolism, cellular lipid status, and lipid biosynthesis.[15,69,74,75] For example, gene expression was evaluated on HepG2 cells treated for 24 hours with 100 mg/mL of SNPs which had a moderate effect on cell viability. Differential gene expression analysis revealed 163 and 230 genes upregulated and downregulated respectively. A network analysis detected TNF, IL4, IGF1, INS, and NOS2 as main local connectivity hubs, while lipidomics analysis showed upregulation of lipid and fatty acid metabolites. Central pathways affected by these particles were those involved in hypercholesterolemia, cholesterol biosynthesis, SREBP signaling, and steroid biosynthesis. Due to the increasing evidence of nanoparticle influence on lipid metabolism and biosynthesis one could

hypothesize that depletion of the membrane and lysosomal overload lipids, resulting from nanoparticle endocytosis, trigger overexpression of genes related to lipid metabolism and biosynthesis. Endocytosis, cellular uptake and lysosomal activation followed by autophagy in response to SNPs is well documented.[76,77] Overstimulation of endocytosis is suggested as an indicator of cytotoxicity of nanoparticles.[78] High endocytosis rate results in cellular stress associated with oxidative stress, mitochondrial dysfunction and autophagy induction.[78] Also, lysosomal vacuolar H+(V)-ATPase genes mediating lysosome acidification by a membrane-associated protein complex upregulation by SNPs were observed.[15,69] SNP accumulation in lysosomes leads to increased membrane permeabilization and lysosome destabilization.[79] Lysosomal dysfunction by SNP accumulation due to particle overload and delayed clearance results in impaired autophagy-mediated protein turnover and cell viability.[80,81]

5.2 Inflammation

Inflammation is the immune system mediated response to invading pathogens and tissue damage. Inflammation can be acute or chronic.[82] Acute inflammation can be beneficial to the host because it results in pathogen elimination and stimulates tissue regeneration. Chronic inflammation, however, is deleterious to the host and is associated with a variety of disorders affecting cardiovascular, nervous, digestive and many other body systems.[83–85] During the inflammatory response, the cells of the immune system produce secondary messengers such as cytokines, leukotrienes, reactive oxygen species (ROS), reactive nitrogen species (RNS), and eicosanoids.[82] These molecules can stimulate other cells in the body to produce a variety of other messenger molecules and growth factors.[82] ROS, produced by neutrophils and macrophages during respiratory burst, and nitric oxide, a form of RNS are among the best studied microbicidal molecules. However, when inflammation is not timely and adequately resolved, these products accumulate in the tissues surrounding the inflammatory site and lead to systemic changes which may affect both deoxyribonucleic acid (DNA) and proteins. For example, some messengers, such as superoxide radicals and RNS, can directly interact with DNA and modify proteins involved in DNA repair. Nitric oxide (NO), nitrogen dioxide (NO2), and peroxynitrite (ONOO-) have been shown to deaminate DNA nucleotides, modify and alter the function of DNA glycosylase and other enzymes involved in the DNA reparation.[86] Likewise, ROS and superoxide radicals (O_2^-) interact with DNA bases.[14, 87, 88] Inducted ROS and corresponding decreases in glutathione are shown to lead to mitochondrial damage and cellular necrosis.[89–91] Excess ROS is believed to induce DNA lesions, mutations, and genomic instability.[92–94] The double membrane of mitochondria is packed with polyunsaturated fatty acids (PUFA). The ROS interaction with PUFA leads to the creation of lipid peroxides which in turn increase membrane permeability, inducing mitochondria swelling.[95,96] The spectrum of products and adducts resulting from oxidation of guanine, lipids, DNA and carbohydrate, nitrosative DNA damage and DNA halogenation commonly detected at the site of inflammation have been described before.[97] Inflammation-mediated genotoxicity may become systemic and affect the immune cells themselves.[98] For example, chemically induced intestinal inflammation leads to DNA damage both in epithelial cells at the site of the inflammation and in circulating immune cells.[98,99] Interestingly, T-cells are affected the most, likely due to their

proliferating function. The DNA damage observed in these cells included both single and double-stranded breaks and oxidative base damage.[99]

Since inflammation may have both direct (e.g., DNA adduct formation) and indirect (e.g., altering the function of the reparation enzymes) effects on genotoxicity, evaluation of nanoparticles pro-inflammatory properties helps to establish the immunological safety profiles of these materials and contributes to the understanding of the mechanisms of nanoparticle genotoxicity. Several reports demonstrate that lung inflammation caused by graphene oxide, combustion particles, diesel exhaust particles and other airborne air-polluting particles is associated with increased levels of oxidatively damaged DNA.[100–102] However, interpretation of the results of such studies is not straightforward, since environmental materials commonly contain other biological (e.g., endotoxin) and chemical (e.g., polycyclic aromatic hydrocarbons) substances leading to inflammation and inducing DNA damage.

5.2.1. The direct contribution of inflammation to SNP genotoxicity—Available studies suggest the association of SNP toxicity with their ability to stimulate the formation of reactive oxygen species (ROS).[103, 104] Silica particle size-dependent ROS synthesis and enhanced oxidative-stress-induced cytotoxicity is widely reported in the literature.[38,69,105–107] Induced oxidative stress by SNPs is the main contributor to DNA damage and mitochondria dysfunction. Systemic administration of amorphous silica nanoparticles (Levasil®) was found to induce both DNA damage and inflammation in rats.[51] The affected organs included liver, lung and peripheral blood leukocytes.[51] Interestingly, particle-size dependent difference was observed in the levels of inflammation and the degree of genotoxic effects. Inflammation, as assessed by plasma levels of cytokines (TNFa and IL-6), was observed with both small and large particles. However, the results were more pronounced in animals treated with silica nanoparticles of a smaller nominal size (15 nm) than with larger particles (55 nm).[51] The genotoxic effects were detected only in animals treated with small size silica nanoparticles.[51] Another study also reported size-dependent induction of ROS and DNA double-stranded breaks by colloidal silica nanoparticles.[108] However, in this study, smaller particles were less toxic.[108] Since both studies did not include mechanistic investigation, the available data do not allow establishing a cause-effect relationship between silica nanoparticles mediated inflammation and genotoxicity. Likewise, other available studies report either genotoxic effects of silica nanoparticles[5,40–43] or their pro-inflammatory properties.[107,109–111] In addition to ROS production and inflammatory response, cytotoxicity of SNPs is linked to cell membrane integrity disruption[103,104,112], disturbed cellular calcium homeostasis[113], aberrant clusters of topoisomerase I (topo I) formation, endothelial dysfunction, and fibrogenesis in Wistar rats.[3,54,114–117] Figure 4 summarizes the mechanistic role of ROS and RNS induction by SNPs in cytotoxicity of these materials. A broader overview of SNP toxicity is available elsewhere.[118–122]

Currently, there is no clear link between genotoxicity and inflammation triggered by silica nanoparticles, which warrants more research in this area. Factors that need to be considered when designing such studies include animal model or cell type, dose levels, dosing regimen, route of administration, a method of particle solubilization/dispersion, and thorough physicochemical characterization. The latter, in addition to the particle physicochemical

parameters (such as size, surface area, zeta potential) should also assess the presence of biological and chemical impurities because these contaminants may confound the results of toxicological studies.[123,124]

5.2.2 SNP effects on expression of inflammation-associated genes—The pattern recognition theory is frequently used to describe the immune response to microbial pathogens.[125] According to this theory, pathogen-associated molecular patterns (PAMPs,) are recognized by their respective pattern recognition receptors (PRR), activation of which triggers inflammatory gene expression.[125] PRRs are expressed in different cellular compartments to provide host surveillance against microbes and tissue damage. TLRs detect PAMPs in close proximity to the cellular membrane and internalized into endosomes[125], while NOD-like Receptors (NLRs) do so in the cytosol.[126] Bacterial lipopolysaccharide (LPS), CpG DNA and antiviral compounds imiquimod and resiquimod are known ligands to some of these receptors.[125] In contrast, nanoparticles are commonly discussed in the context of so-called “sterile” inflammation.[127,128] According to this model, cell death and tissue damage in response to mechanical, physical or chemical stress results in the generation of the so-called damage-associated molecular pattern (DAMPs) or danger signals.[127, 128] Examples of DAMPs include high mobility group B1 (HMGB1) protein and uric acid crystals.[129] DAMPs are also recognized by the set of innate immune receptors which activate the inflammatory response. Some PRRs serve as receptors for both PAMPs and DAMPs. For example, bacterial LPS and HMGB1 can both activate Toll-like Receptor 4 (TLR4).[130,131] A term nanoparticle-associated molecular patterns (NAMPs) has also been coined to distinguish nanoparticle recognition from DAMPs and PAMPs.[132] However, there is no clear structural distinction between NAMPs and DAMPs. Moreover, some nanoparticles are recognized both by PAMPs- and DAMPs-sensing PRRs. For example, silica nanoparticles are sensed by scavenger receptors,[133] while stress triggered by these nanomaterials generates HMGB1 release sensed by TLR4.[134] Thus, classification on DAMPs and NANPs is valid only based on the origin of the cell damage initiating signal and has little functional utility.

NLRP3 inflammasome, a complex of proteins assembled in the cytosol, plays a critical role in the secretion of cytokines of interleukin 1 (IL-1) family which includes IL-1β, IL-18, and IL-33.[135,136] Many fibrous, cationic and crystalline nanoparticles activate NLRP3 inflammasome through lysosomal damage.[137–139] Such damage occurs as a result of either mechanical disruption (e.g., in the case of titanium nanobelts)[137] or proton-sponge effects (e.g., cationic liposomes).[138,139] It is essential to keep in mind, that induction of cytokines associated with the activation of NLRP3 pathway requires two signals: signal 1 – to activate the gene expression and production of a precursor protein, and signal 2 – to activate caspase 1 to cleave the precursor and release mature cytokine. Although NLRP3 inflammasome activation has been reported for both crystalline[133,140] and amorphous silica,[117,141] production of IL-1 family cytokines in response to these materials implies the presence of additional PAMPs or DAMPs which provides the signal. Bacterial endotoxin, a common contaminant in engineered nanomaterials may provide such signal.[123] Therefore, interpretation of studies demonstrating the induction of IL-1 by silica nanoparticles critically

depends on the characterization data regarding the endotoxin content in these nanomaterials. Such information is frequently omitted in the published studies.

Iron-coated silica nanoparticles with mean diameter of 60±12 nm and surface area of 56.15±0.35 m2/g were exposed to human THP-1 macrophages to investigate pro-inflammatory effects and antioxidant gene induction; the analyses were performed by real-time PCR and western blot assays.[142, 143] mRNA measurements revealed time- and dose-dependent induction of TNFα and IL-1β gene expression by the particles; the response started at 3 hours of exposure and continued up to 9 hours. At the later time points, starting at 9 hours and up to 18 hours of exposure, the study addressed the effects on the anti-oxidant response. Such analysis included evaluation of the nuclear factor erythroid 2 -like 2 (Nrf2), a transcription factor regulating expression of antioxidant genes required for the cellular response to environmental stress such as exposure to oxidative stress-inducing nanomaterials.[144,145] Nrf2-regulated genes and proteins (GCLC, GCLM, HO-1, and NQO-1) were also assessed. This study established a relationship between inflammation triggering NF-κB pathways, and anti-oxidant producing Nrf2 signaling activated in response to SNPs.[143]

Microarray gene expression profiling of A549 cells treated with commercially available Ludox® silica nanoparticles of different sizes (SM30 and AS30) identified genes contributing to cytotoxicity. Despite comparable cytotoxicity between these particles, gene-by-gene, and gene set analyses revealed a statistically significant up-regulation of matrix metalloproteinases (MMP1, MMP10, and MMP9), TNFα, IL1β and ATM genes by smaller (SM30) SNPs; among these genes, MMP1 and MMP9 showed the most dramatic upregulation.[14] Previously it was shown that air pollutants such as diesel exhaust particles induce matrix metalloproteinases in A549 cells.[146,147] Also, *MMP9* induction via TLR/MyD88 cascade was observed and suggested as a triggering point of apoptosis mediating *MMP9* for extracellular matrix protein degradation.[148–150] Both of these metalloproteinases are also known as DAMPs. Therefore, expression of these genes in response to particle exposure confirms stress and damage effect of SNPs on these cells.

Acute inflammation accompanied by secretion of pro-inflammatory cytokines (TNF, IL-1, IL-8, and IL-6) and chemokines (MIP-2 and MCP-1) was reported *in vivo* after intratracheal instillation of silica nanoparticles in mice.[109] Similarly, elevation in levels of pro-inflammatory cytokines (IL-1 and TNF) was observed in the blood of mice after i.p. injection of silica nanoparticles.[110] The same study reported an increase in the numbers of NK and T-cells and a decrease in the number of B-cells in spleens of the treated animals as compared to the control animals.[110] Change in the inflammatory gene expression in response to SNPs was observed *in vivo* using alternative models as well. For example, neutrophil-mediated cardiac inflammation, down-regulation of cardiac muscle contraction genes (atp2a1l, atp1b2b, atp1a3b), and also down-regulation of calcium channel-related genes (cacna1ab, cacna1da), leading to cardiac dysfunction were further demonstrated in a zebra-fish model.[151]

5.2.3. SNP-mediated activation of genes involved in oxidative burst—

Oxidative burst in response to silica nanoparticles has been reported in a variety of cell lines,

including murine macrophages (RAW 264.7),[110] human lung cancer A549 [112] and neuronal cells.[108] These responses were dependent on particle charge and size. Induction of pro-inflammatory chemokines (IL-8 and MIP-2) were observed in response to silica nanoparticles in both immune (e.g., alveolar macrophages) and non-immune (e.g., fibroblasts) cell lines.[114,152,153] When silica nanoparticles were incubated in the presence of antioxidants, secretion of MIP-2 chemokine was inhibited,[111] suggesting a mechanistic link between ROS generation and chemokine secretion.

Gene expression and metabolic changes of human embryo kidney 293 (HEK 293) cells incubated for 12 hours with 0.1 and 10 ug/mL of 50 nm Rhodamine B isothiocyanate labeled (RITC) magnetic cobalt ferrite core ($CoFe_2O_4$) and silica shell nanoparticles (MNPs@SiO_2(RITC)) were investigated using microarray and gas chromatography-mass spectrometry. Cytotoxicity in HEK 293 cells was not observed after 7 days of treatment.[154] In addition, no toxicity in human cord blood-derived mesenchymal stem cells up to 0.1 μg/μL MNPs@SiO_2(RITC), and no genotoxicity in Chinese hamster lung fibroblast cells up to 1.0 μg/μL were detected.[155,156] Transcriptome profiles revealed altered expression of 291 genes, 205 and 86 of which were upregulated and downregulated, respectively.[154] Metabolite profiling analysis showed increased glutamic acid levels. Several genes such as GAD1, GOT2, GLUD1, and GLUL, known to be involved in glutamic acid catabolism, were downregulated, however, ALDH4A1 and GPT2 genes involved in glutamic acid synthetic transaminase were upregulated. Intracellular ROS increase, induction of mitogen-activated protein kinase (MAPK) pathway, and damage to mitochondria were also detected.[154] This study concluded that despite an increase in the ROS levels, elevated amounts of glutamic acid prevented oxidative stress-mediated cell death by inducing increased antioxidant levels.

In another study RNA sequencing demonstrated 42 up-regulated and 77 down-regulated differentially expressed genes in C17.2 cells treated with SNPs at a concentration of 200 μg/mL. Among ten pathways recognized by KEGG analysis, three main pathways were identified by pathway-act-network and included the MAPK signaling pathway, apoptosis pathway, and the PI3K-Akt signaling pathway. Interestingly, glutathione-S-transferase genes (GST) (GSTM1, GSTM7, and GSTT1) were down-regulated. GSTs gene family is involved in conjugation with glutathione to xenobiotic substrates as a detoxification enzyme. Mitochondria swelling, dysfunction, and mitochondrial cristae deformation in C17.2 cells exposed at 200 μg/mL were observed and related to the induction of the oxidative stress.[118]

When SNPs are used as drug delivery vehicles,[157–159] alteration in gene expression profiles in response to the carrier can be further influenced by the drug. For example, cytotoxicity studies demonstrated the significantly higher potency of drug loaded MSNs as compared to the free drug and the free particle carrier tested separately. Enhanced drug efficacy of combined drug and MSN system was attributed to intracellular drug release and potentially newly activated pathways.[160, 161] DNA microarray analysis was applied to explore gene expression profile of HeLa cells exposed to 40–50 nm MSNs, drug doxorubicin (DOX), and DOX-loaded MSNs in an attempt to gain mechanistic insights into cell death.[96] Interestingly, the cell death mechanism of free drug was attributed to apoptosis, while that of the MSN-delivered DOX-induced both apoptosis and direct necrosis.[96] Differential expression of 3, 152, 3, 180, and 297 genes was observed in HeLa cells exposed for 24 hours

to DOX-loaded MSNs, free DOX, and MSNs respectively. At the tested concentration (80 μg/mL) 90, 45, and 35% viability was observed in cells treated with MSNs, free DOX and DOX-loaded MSNs, respectively. Although alteration of expression of genes involved in stress, cell cycle, inflammation, RNA processing, metabolic process, cell death, and apoptosis was observed in both free DOX and MSN-DOX, the effects were more pronounced in cells exposed to the DOX-loaded nanoparticle. KEGG analysis also revealed lysosome pathway upregulation in cells treated with DOX-loaded MSN.[96]

6. The role of long-term toxicity studies in understanding genotoxic potential of SNPs

Silica nanoparticles seem to be safe regarding cellular genomic response for drug delivery applications at dosages below MTD. However, challenges of inefficient loading capacity, low accumulation in tumor site, and dosimetry should be addressed to obtain successful therapeutic effects. In a potential best case scenario, hollow structured MSN has a maximum of 10% drug loading capacity. Small portion of injected SNPs loaded with drug accumulates in the tumor site. Only 6% of the injected dose/g tissue in tumor after 24 h has been observed.[162–164] Successful drug delivery for tumor growth inhibition could be limited for SNPs as a platform for cancer therapy with single dose administration due to corresponding limitations. To have effective inhibition of tumor growth, repeated administration of formulated SNP drug carriers below MTD is required, since equivalent single dose injection would be an order of magnitude higher than MTD and would be considered toxic. Repeated administration of SNPs can lead to chronic exposure. Despite numerous studies on acute toxicity, little is known about the effects of chronic exposure of SNPs. Chronic inflammation, carcinogenic potential, tumorigenesis, increased genetic instability, and genomic responses as primary and secondary effects of chronic exposure to SNPs should be studied in more depth. The dissolution of amorphous silica in vitro and in vivo as function of particle physicochemical also merits detailed investigation.

Depending on the route of administration, nanotoxicity studies revealed cell type-, time-, and dose-dependent toxic effects of SNPs with certain physicochemical properties. The studied toxicities include damage of cells,[17, 165, 166] tissues, and organs.[167–169] Despite numerous studies on acute toxicity, there are limited investigations of the effects of chronic exposure of SNPs. A shortcoming of experimental models prevents studies of chronic inflammation and carcinogenic potential of SNPs. Although previous investigations revealed SNPs capability to induce ROS generation, autophagy, inflammation, DNA damage, and DNA strand breaks, there are limited number of studies which investigate the influence of synthesized SNPs on inflammation, genotoxicity, and carcinogenesis or tumorigenesis in chronic exposure settings. Low-dose chronic exposure to amorphous SNPs was studied in BEAS-2B human lung epithelial cells[170]. In this experiment cells were cultured for 40 passages in the presence of 5 μg/mL negatively charged SNPs of 57.66 ± 7.30 nm average diameter. A malignant transformation such as increased cell migration, cellular proliferation, and anchorage-independent cell growth were detected. SNP-transformed cells were tumorigenic *in vivo* in a xenograft nude mouse model. Genome-wide transcriptional analysis and signal-net analysis revealed p53 signaling as a principal signaling mechanism of

malignant transformation and carcinogenicity of amorphous SNPs.[170] Loss of growth-arresting signals, increased genetic instability, epigenetic alterations, and inappropriate cell survival, as a result of loss or inactivation of p53 gene, contributed to the carcinogenicity of amorphous SNP chronic treatment.[5, 151, 171] Although, the follow up study demonstrated tumorigenicity of the cells exposed to SNPs, we need to keep in mind that the follow up study was conducted in immunocompromised animals which do not accurately represent actual life scenario. Nevertheless, this study demonstrates the utility of the long-term *in vitro* exposure to nanomaterials as a tool to reveal potential genotoxic and carcinogenic effects of SNPs. Additional *in vivo* studies employing chronic exposure of animals to nanoparticles are required to verify the initial findings obtained using such combination of *in vitro* and *in vivo* xenograft models.

7. Conclusion and future directions

There are limited studies on genotoxicity of amorphous SNPs. Conflicting results have been reported, and the field suffers from lack of conclusive studies. Despite recent efforts to answer questions and address problems in SNP nano-genotoxicity, comprehensive studies are required to identify genotoxicity levels, mechanism of genotoxicity, the prolonged influence of particle exposure, the role of the immune response, the impact of physicochemical properties, and route of exposure. One major problem in interpreting SNP genotoxicity data is an abundance of variable influential parameters that have to be controlled one by one to clarify the impact of each factor on genotoxicity. Parameters changing genotoxic outputs of SNP assessment can be grouped into 2 major classes: 1) physicochemical properties of particles such as size, charge, agglomeration state, porosity, surface area, geometry, surface properties, degradability, dissolution, and density among others; and 2) treatment conditions such as concentration, dosimetry, medium, exposure time, cell type, animal model, and single or consecutive treatments.

A systematic approach wherein thorough physicochemical characterization, including detection of chemical and biological impurities in silica nanoparticles, and biological studies, including *in vivo*, *ex vivo* and *in vitro* end-points to address multiple pathways and toxicity types, is needed. One way to achieve this is by leveraging technologies such as organs-on-a-chip cultures,[172] mass-cytometry[173] and dynamic simulation analysis.[174] New emerging methodologies such as next-generation RNA sequencing (RNA-Seq) for global high-throughput gene expression quantification and investigating the genotoxic effect of nanoparticles can be utilized to uncover underlying mechanism and pathways of cytotoxicity and genotoxicity. Whole-genome analysis investigates transcriptionally controlled processes associated with nanoparticle exposure, providing new clues on the mechanism(s) of toxicity. While each specific cellular assay provides a single snapshot of cell function, RNA-Seq depicts a precise overall state of the cell, providing insight into the signaling pathways and cascades of events. Time- and dose-dependent cell response to SNPs can be confirmed with hierarchical stress response as membrane trafficking followed by inflammatory response and acute oxidative stress. Studies conducted by gene expression analysis suggests ROS production by amorphous SNP uptake may not be the only mechanism of cytotoxicity.

Further investigation is necessary to fill the existing research gaps, improving the understanding of mutagenicity of SNPs, particles' possible direct or indirect effects or secondary interactions, and the role of ROS on induction of genotoxic response. Future studies can focus on evaluating the impact of SNPs on cell-dependence of genotoxicity. Current literature is limited to short-term acute genotoxicity studies. Treating *in vitro* or *in vivo* models beyond weeks and months in consecutive time points would provide additional understanding of accumulation, inflammation, clearance, and immune system role in the potential genotoxic response. Almost all studies involved SNP genotoxicity assays for a short period for which secondary impacts of SNPs such as induction of inflammatory response, oxidative stress, and immune system responses in the induction of genotoxic effect have not been investigated. Non-standard methodologies used in various laboratories for nano-genotoxicity examination without validation can lead to further contradictive results. An established protocol indicating a series of experiments in tandem (e.g., comet assay, micronucleus assay, RNA expression) and clarifying conditions of each experiment (dosing, concentrations, time intervals) are required to improve the quality of genotoxicity studies. None of the at hand assays alone are capable of accurate assessment of particle genotoxicity to facilitate general conclusions.

Another future direction for cytotoxicity or genotoxicity of SNPs is the "co-exposure system" studies that have been neglected so far. There are scarce studies on co-exposure systems of SNPs and drugs or toxic moieties. Considering large production volume and a wide range of commercial applications of SNPs, most probably particles interact with co-existing contaminants. Combined SNPs with drugs or toxic moieties could have a potentially synergistic effect. The cytotoxicity or genotoxicity of SNPs could be magnified due to unique properties of nanosize silica particles. Unmodified SNPs on average have 4 to 5 OH/nm^2 silanol groups on the surface. Chemical moieties with higher affinity to hydroxyl groups could potentially be adsorbed on SNPs. Considering the large surface area of SNPs, adsorption of toxic chemical moieties could result in increased concentration of contaminants for which co-exposure of SNPs and toxic moieties could induce toxicity at low levels that either SNPs or chemical moiety alone could not induce.[175–178] For example, zebrafish embryos co-exposure to SNPs and benzo[a]pyrene (B[a]P) has been studied at very low concentrations (No Observed Adverse Effect Level). While neither SNPs nor benzo[a]pyrene (B[a]P) could induce cardiac toxicity phenotype at the low concentrations in zebrafish embryos, the co-exposed system of SNPs and B[a]P significantly induced pericardial edema and bradycardia.[175]

Despite significant studies on acute toxicity, there are inadequate investigations on longterm (half year to one year and beyond) genotoxicity and gene expression profile by amorphous silica nanoparticles. Repeated administration of SNPs could result in chronic exposure leading to chronic inflammation. Chronic inflammation is known to induce a variety of disorders affecting cardiovascular, nervous, digestive and many other body systems. There are limited number of studies assessing SNPs impact on inflammation, genotoxicity, and gene alteration in chronic exposure settings. There is great need for in depth evaluation of increased genetic instability, carcinogenic potential, and chronic inflammation induced by chronic exposure to SNPs.

We envision that future directions for genotoxicity studies of SNPs will focus on the following areas. First, comprehensive systematic experiments should be done to investigate the role of physicochemical properties of SNPs on induction of genotoxic response in different cell lines and animal models. Second, long-term toxicity and effects of consecutive SNP exposure on health should be evaluated. Third, one has to establish a standardized and comprehensive protocol for evaluating genotoxicity as a function of route of exposure. Fourth, co-exposure systems of SNPs and drugs or chemical moieties in the context of the genotoxic study to be included in study design. Fifth, time- and dose-dependent studies are needed to uncover course of action and direction of gene regulation. Finally, the studies should evaluate the utility of new emerging methodologies, such as RNA sequencing method, to assess global gene expression and genotoxic effect with the aim of determining mechanism and pathways of genotoxicity. It must be noted that such studies would need to be done in the context of specific intended or unintended exposures and with relevant physicochemical properties and concentrations to provide meaningful results for the use of SNPs in biomedical applications, consumer products, or in circumstances where unintended environmental exposure is encountered.

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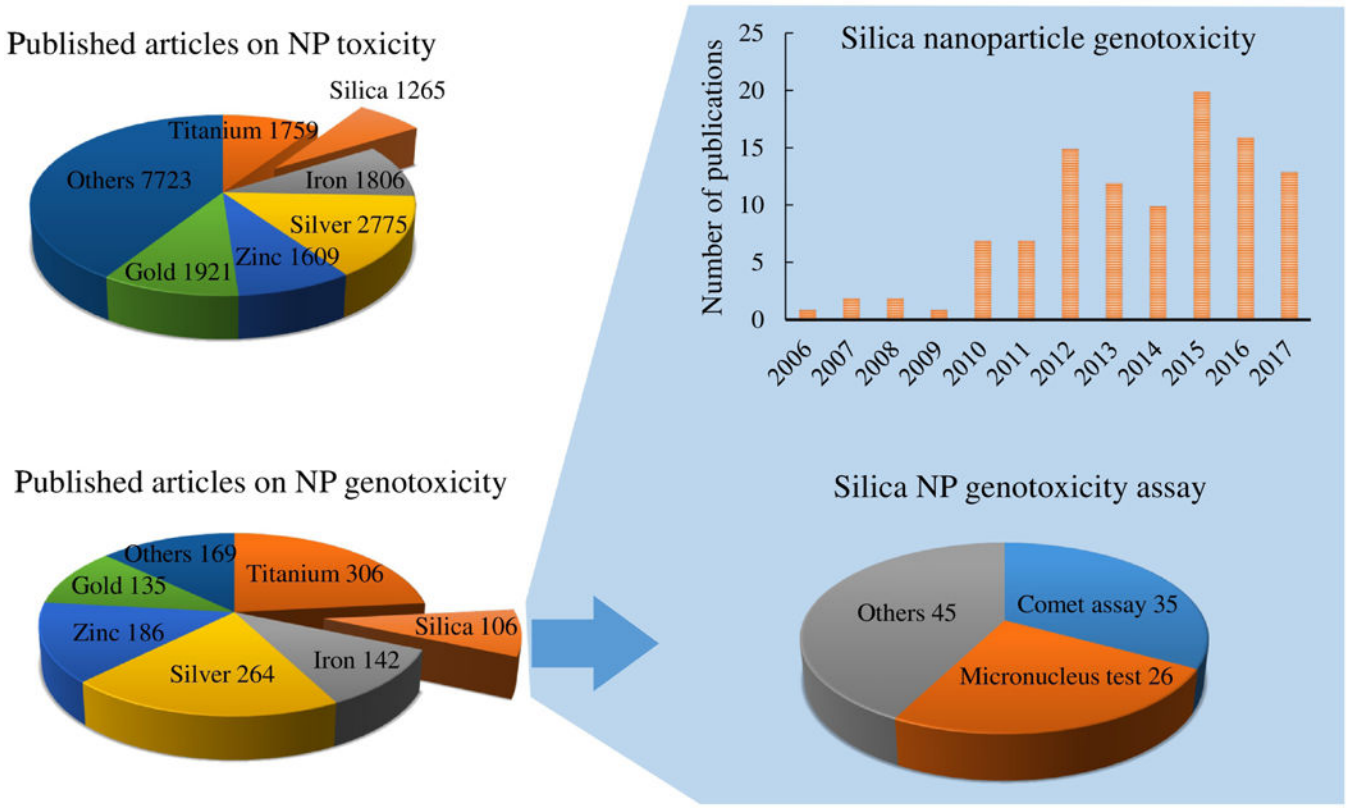

Figure 1.

Summary of observed trends of the influence of SNP physicochemical properties on *in vitro* and *in vivo* toxicity.

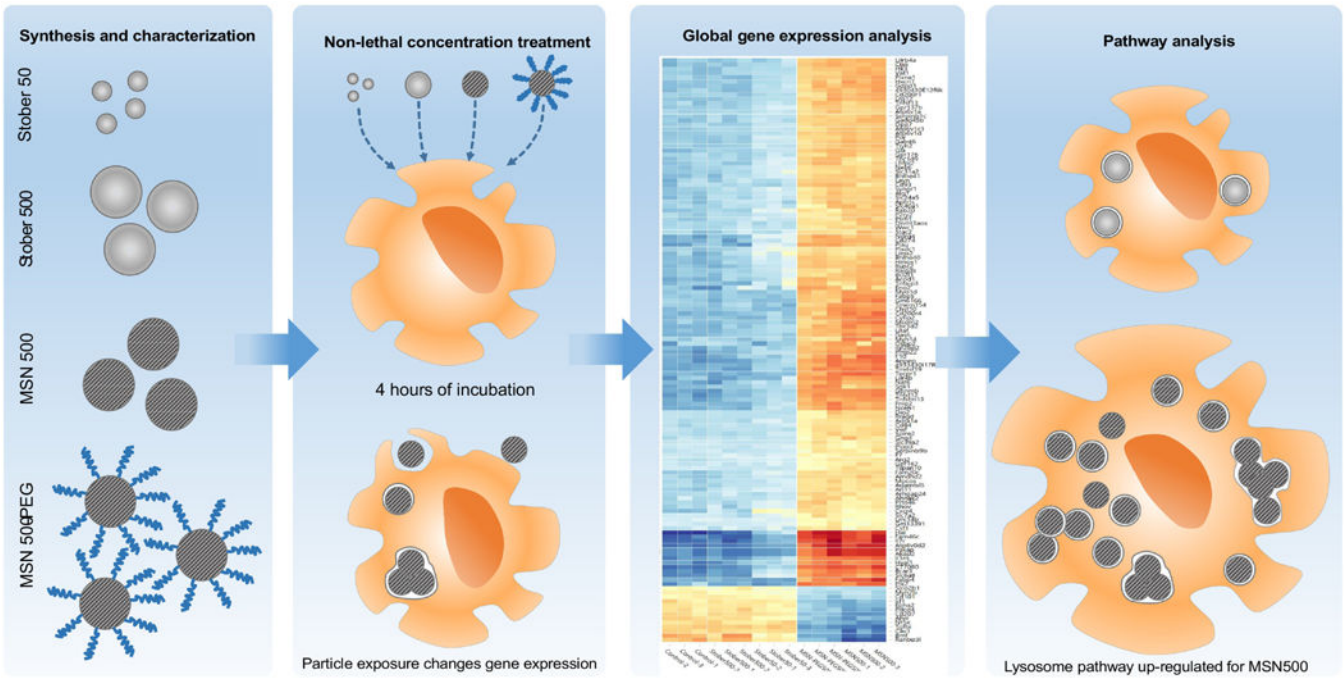

Figure 2.

Literature survey on NP toxicity and genotoxicity using “NPs and toxicity,” “NPs and genotoxicity,” “SNPs and genotoxicity and comet assay” and “SNPs and genotoxicity and micronucleus assay” kewords in the Scopus database.

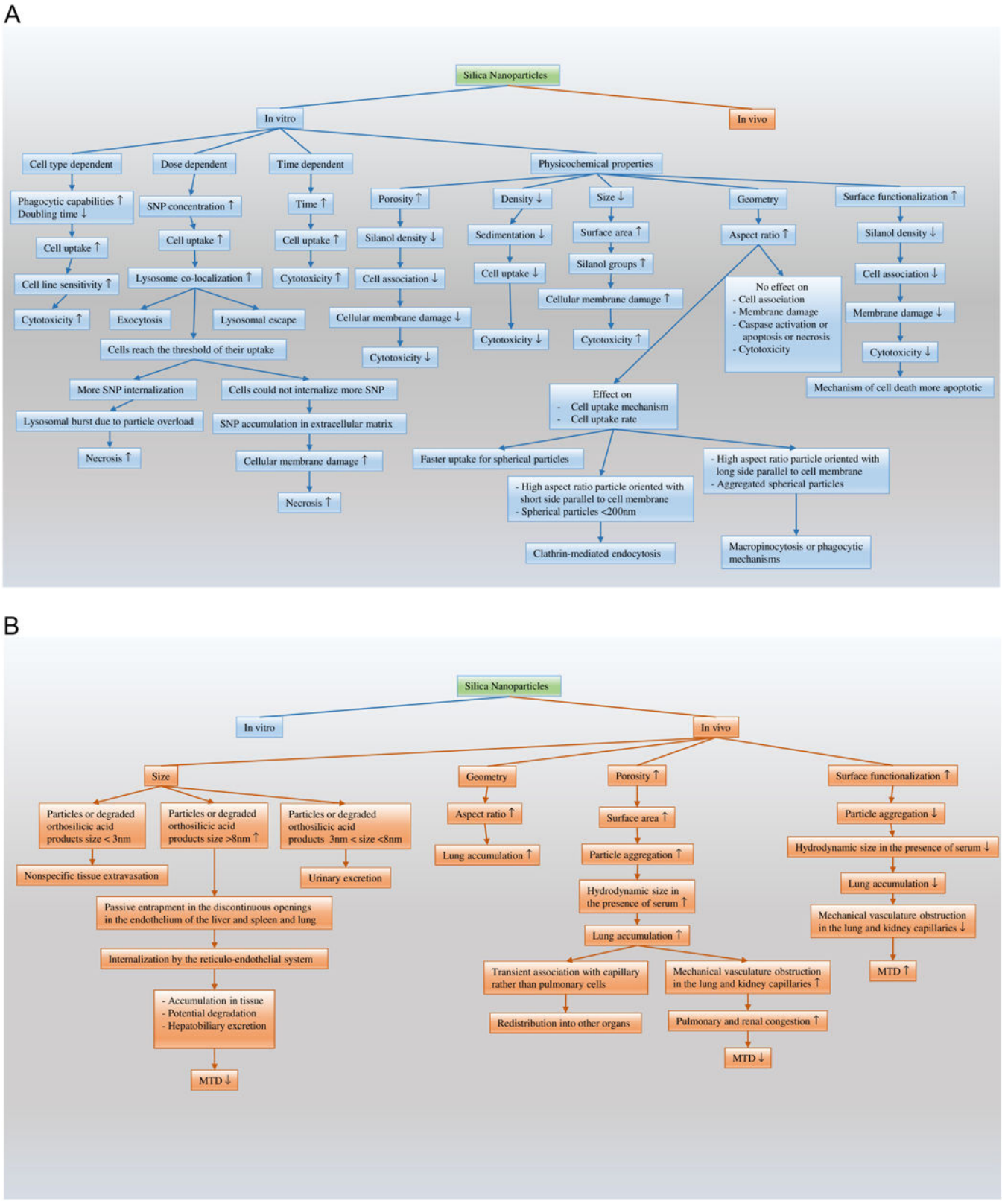

Figure 3.

Spherical nonporous and mesoporous SNPs of different diameters and PEGylated mesoporous SNPs exposed to RAW264.7 macrophages at sub-toxic does for four h. RNA-sequencing generated transcriptional profiles, and differentially expressed genes were analyzed by GATHER and DAVID software. Only mesoporous SNPs exhibited gene expression alteration related to lysosomal activation, in the absence of intracellular ROS production, mitochondrial damage, and mitochondrial membrane potential disruption (with permission from reference 15).[15]

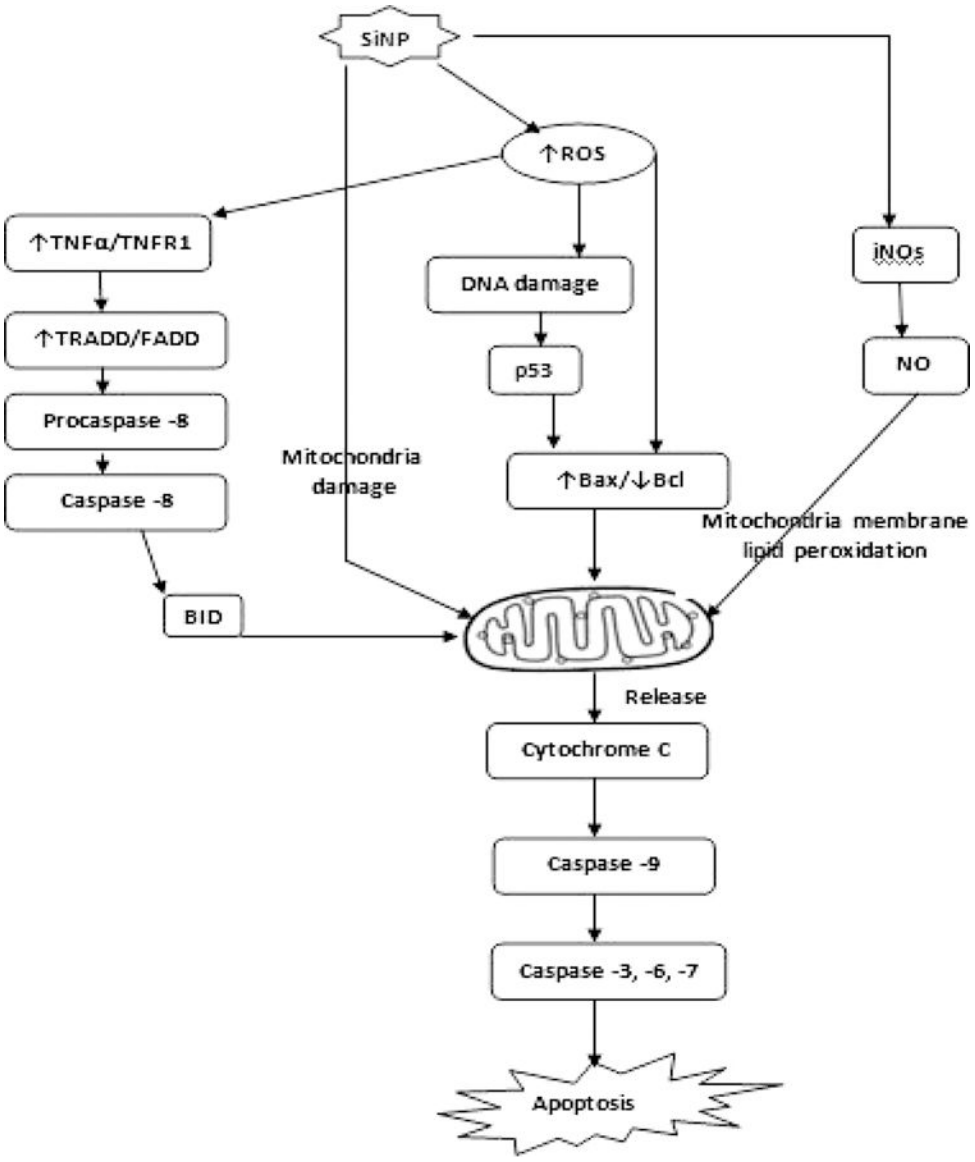

Figure 4.

Mechanisms of cytotoxicity induced by SNPs through ROS generation, directly interacting with mitochondria, or nitric oxide related pathway (with permission from reference 118).[118]

Table 1

Summary of published SNP genotoxicity studies

Nanoparticle	Physicochemical characterization	Result of characterization	Cells/organism	Test	Treatment conditions	Result	Reference
Alumina-coated SNPs (laboratory synthesized) and Ludox® colloidal silica (Sigma-Aldrich)	Average particle sizes and shapes (TEM) DLS (hydrodynamic radius of particles and dispersion/ aggregation) Zeta potential (mV)	33.21, 34.89, 240.08, 21.32, 30.51 nm 77.9, 65.9, 3269,46.2, 20.5 nm −33.79, −10.6, −49.1, 50.7, −59.2	3T3-L1 fibroblasts	Comet assay	3, 6, and 24 h incubations and 4 or 40 μg/ml	No significant genotoxicity was observed	59
Spherical amorphous SNPs	TEM energy-dispersive X-ray spectroscopy (EDX) DLS	14 nm Agglomerate in DMEM (500nm)	Epithelial cell lines of HaCat, HT29, and A549	Comet assay	Concentrations up to 10 μg/ml serum-free DMEM incubation for 24 h	Significant DNA damage	66
Five unlabeled and one fluorescently-labeled SNPs	Size distribution and morphology (SEM and DLS)	25 ± 7.3, 16.4 ± 2.9, 38 ± 24, 70 ± 14 nm	Balb/3T3 mouse fibroblasts	Cell Transformation Assay (CTA) Micronucleus (MN)	1 μg/mL, 10 μg/mL and 100 μg/mL Incubation times up to 72 h	SNPs are internalized in cells, no cytotoxic or genotoxic or morphological transformation	3
Ludox® colloidal silica (Sigma-Aldrich)	TEM DLS Zeta potential	Spherical shape No agglomerations 6±0.6, 15±0.9, 30±0.4, 55±0.7 nm −45.1, −69.9, −54.1, −54.3 mV	Human peripheral blood lymphocytes Human embryonic kidney (HEK293) cells	Comet assay with and without the Fpg and Endo III enzymes	1 μg/mL, 10 μg/mL and 100 μg/mL	Significant induction in DNA damage at 100 μg/ml dose-dependent genotoxicity	65
Spherical amorphous SNPs (Glantreo, Ltd, Ireland)	TEM Zeta potential DLS	11 nm (ξ −43.3 mV), 34 nm (ξ −33.7 mV), 34 nm (ξ −10.6 mV), and 248 nm (ξ −49.1 mV) 103.1 ± 1.9, 77.9 ± 1.1, 65.9 ± 1.7, 269.0 ± 7.1 nm	3T3-L1 mouse fibroblasts	Mouse embryonic fibroblasts carrying the lacZ reporter gene (MEF-LacZ) Micronucleus (MN)	24 h at concentrations of 4, 40 or 400 μg/ml	Gene mutations and chromosomal aberrations induction for 34 nm SNPs	5
Ludox® colloidal silica (Sigma-Aldrich)			MCF-7 (human breast cancer cell line)	Comet assay	4 μg/L and 40 μg/L	Nanoparticle suspensions are genotoxic in human cells	64
Luminescent SNPs (laboratory synthesized)		50 ± 3 nm in diameter	Human lung epithelial cells (A549)	Comet assay DNA repair enzyme activity DNA agarose gel	Up to 100 μg/ml concentration Incubation times up to 72 h	No significant increase in DNA repair activity or DNA damage	67
Aminated and non-aminated SNPs	TEM hydrodynamic diameter and zeta potential in RPMI-1640 medium, and Bovine Serum Albumin (BSA 0.1 %), and lung lining fluid (LLF 1 lg/ml) and serum 0.1 %)	50 and 200 nm in diameter Size and charge differ in different media	Human lung epithelial cells (A549)	Comet assay MN	The concentration of 62.5 μg/ml incubated for 4 h for Comet assay Incubated for 24 h for MN assay	Size-dependent DNA damage by comet assay Reduced DNA damage when the particles were dispersed in BSA or serum MN assay did not show genotoxicity	68
Spherical amorphous SNPs (E&B Nanotech Co Ltd Republic of Korea)	Field emission scanning electron microscope (FESEM) Zeta potential DLS	33 and 90 nm in diameter 23±0.1 nm and 91.6±0.5 nm hydrodynamic size −40 mV surface charge in distilled water	*S. typhimurium (TA98, TA100, TA1535, and TA1537) and in E. coli WP2uvrA* Chinese hamster lung (CHL) fibroblast cells Male Crl: CD (Sprague Dawley) rats ICR mice	Bacterial reverse mutation test *In vitro* chromosome aberration test *In vivo* comet assay and micronucleus test	313, 625, 1250, 2500, and 5000 μg per plate 175, 350, 700, and 1400 μg/mL 500, 1000, and 2000 mg/kg body weight	No statistically significant genotoxicity	72

Nanoparticle	Physicochemical characterization	Result of characterization	Cells/organism	Test	Treatment conditions	Result	Reference
Spherical and rod-like mesoporous SNPs (laboratory synthesized)	Size, shape (TEM, SEM), BET surface area	Sphere-shaped 100 nm, 1169 m^2/g Rod-shaped 240 nm, 994 m^2/g	DNA repair deficient cell line (chicken DT40)	Chromosomal aberration analysis	10, 25, 50, 75, and 150 μg/ml concentrations	Mitotic chromosomal aberration Rod-shaped particles showed the higher genotoxic effect	[70]
Amorphous SNPs (Research Centre nanomaterials repository, Italy)	TEM Energy dispersive spectrometry (EDS) Nitrogen Adsorption DLS	18.3 ± 4.5, 18.0 ± 1.0, 17.7 ± 2.5, 24.7 ± 17.7 nm Main impurities (sodium, sulfur, and aluminum) below 1% 189, 140, 204, 204 (m^2/g) 575.0 ± 97.6, 286.6 ± 19.2, 143.3 ± 5.1, 154.7 ± 7.6 nm	Male Sprague Dawley rats	Alkaline and the (Fpg)-modified comet assays MN	Gavage 5, 10, or 20 mg/kg b.w./day for three days at time intervals of 48, 24, and 3 h	No significant DNA strand breaks or damage Slightly increased micronucleation observed in the colon	[73]
Amorphous spherical SNPs Levasil® 200/40% and Levasil® 50/50% (HC Stark, Germany)	Cryogenic transmission electron microscopy (Cryo-TEM) 3D dynamic light scattering (3D-DLS) X-ray diffraction Energy-dispersive X-ray spectroscopy (EDX)	15 and 55 nm mean particle size at pH 7.5 by DLS 31.6 and 105.1 nm	Adult male Wistar rats	*In vivo* Comet assay MN *In vitro* MN assay performed with human blood	Three consecutive intravenous injections Time intervals of 48, 24, and 4 h before tissue collection Dosing at MTD of each particle (50 mg/kg for 15 nm SNPs and 125 mg/kg for 55 nm SNPs)	Reproducible DNA damage and micronucleated reticulocytes observed Tissue damage mediated inflammatory response Secondary genotoxicity mechanism No significant genotoxicity detected by *in vitro* MN assay	[71]
Amorphous SNPs (Research Centre nanomaterials repository, Italy)	TEM Energy dispersive spectrometry (EDS) Nitrogen adsorption DLS	18.3 ± 4.5, 18.0 ± 1.0, 17.7 ± 2.5, 24.7 ± 17.7 nm Main impurities (sodium, sulfur, and aluminum) below 1% 189, 140, 204, 204 (m^2/g) 575.0 ± 97.6, 286.6 ± 19.2, 143.3 ± 5.1, 154.7 ± 7.6 nm	Male Sprague Dawley rats	*In vivo* Comet assay MN	Intratraecheal instillations at three different doses of 3, 6, or 12 mg/kg at time intervals of 48, 24, and 3 h Intravenous injection at three different doses of 5, 10, or 20 mg/kg at time intervals of 48, 24, and 3 h	No significant genotoxic effect	[55]
Aerosolized amorphous SNPs (laboratory synthesized)	Long differential mobility analyzer (long DMA) Nano differential mobility analyzer (nano DMA) Condensation nucleus counter (CNC) Aerosol electrometer (AE) TEM	Particle number concentration 37 or 83 nm	Male Crl:CD (SD)IGS BR rats	*In vivo* MN	Inhalation 1- or 3-day exposures 3.7×107 and 1.8×108 particles/cm^3 (corresponding to mass concentrations of 1.8 or 86 mg/m^3)	No significant genotoxic or pulmonary inflammatory effects	[74]
Amorphous SNPs (Sigma-Aldrich)	Laser correlation spectroscopy (LCS)	15 ± 2 nm in diameter	Human peripheral blood lymphocyte F1 female mice (C57BL × CBA) and outbred Wistar female rats	Chromosome aberration assay MN comet assay	20, 100, and 200 μg/ml concentrations Pre- and post-implantation embryos of mice and rats	No significant genotoxicity in human peripheral blood lymphocyte Impaired embryogenesis	[75]
Amorphous silica spherical SNPs Levasil® (HC Stark, Germany) Microparticulated silicon dioxide (Sigma-Aldrich)	TEM DLS Laser Doppler velocimetry (LDV)	Size distribution and morphology No agglomerations 6, 15, 30 and 55 nm 6 ± 0.6, 15 ± 0.9, 30 ± 0.4, 55 ± 0.7 nm −45.1, −69.9, −54.1 and −54.3 mV	Drosophila melanogaster	Comet assay Wing-spot assay	0.1, 1, 5 and 10 mM concentrations	No significant change in wing-spot assay Dose-dependent DNA damage in larvae No genotoxic effects observed with microparticulated silica particles	[76]