Review

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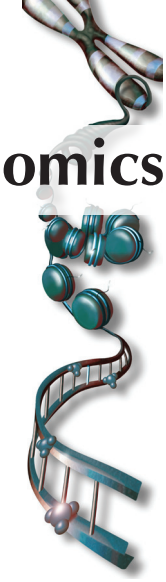
Epigenomics



Epigenetic mechanisms in nanomaterial-induced toxicity

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With the growing advent of nanotechnology in medicine (therapeutic, diagnostic and imaging applications), cosmetics, electronics, clothing and food industries, exposure to nanomaterials (NMs) is on the rise and therefore exploring their toxic biological effects have gained great significance. *In vitro* and *in vivo* studies over the last decade have revealed that NMs have the potential to cause cytotoxicity and genotoxicity although some contradictory reports exist. However, there are only few studies which have explored the epigenetic mechanisms (changes to DNA methylation, histone modification and miRNA expression) of NM-induced toxicity, and there is a scarcity of information and many questions in this area remain unexplored and unaddressed. This review comprehensively describes the epigenetic mechanisms involved in the induction of toxicity of engineered NMs, and provides comparisons between similar effects observed upon exposure to small or nanometer-sized particles. Lastly, gaps in existing literature and scope for future studies that improve our understanding of NM-induced epigenetic toxicity are discussed.

Keywords: air pollutants • chromatin condensation • DNA methylation • epigenetic toxicity • genotoxicity • histone modifications • miRNA • nanomaterial • nanotechnology

The nanotechnology industry has been rapidly growing over the years with promising applications in medicine, engineering and the household. More than 1600 nano-products are currently available, out of which more than 700 are categorized under 'health and fitness' [1]. While the idea of nanotechnology was first introduced by Richard Feynman who proposed precise manipulation of atoms and molecules to create 'nanoscale' machines [2], the term 'nanotechnology' was first coined by Norio Taniguchi in 1974. The term 'nano' is derived from the Greek word 'nanos' that stands for 'dwarf' [3].

The European Commission has defined a nanomaterial (NM) as "*a natural, incidental or manufactured material containing particles, in an unbound state or as an aggregate or as an agglomerate and where, for 50% or more of the particles in the number size distribution, one or more external dimensions is in the size range 1–100 nm*" [4]. The term 'material' refers to 'object' according to the International Organization for Standardization definition, where "*nano-object includes nanoplates (one dimension in the nanoscale), nanorods (two orthogonal dimensions in the nanoscale) and nanoparticles (three orthogonal dimensions in the nanoscale)*" [5]. With a decrease in particle size, the ratio of surface area to volume of the NMs increases, forming the basis for differences in their physico-chemical properties when compared with their bulk material. The large surface area to volume of the NMs increases the number of atoms on the surface of these particles contributing to their increased bio-reactivity and toxicity [6,7].

Toxicity of engineered nanomaterials

The field of nanotoxicology encompasses unravelling the biological effects of engineered nanomaterials (ENMs) [8]. Although nanotechnology has existed since the 1980s,

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studies on the toxic effects of ENMs have gained momentum with the increase in nano-products used in medicine, cosmetics and food [9]. The toxicity of ENMs has been assessed both *in vitro*, by exposing cells to NMs and *in vivo* in various animal models exposed to NMs.

Factors critical for determining nanotoxicity

NMs are taken up by cells through diverse mechanisms, namely, endocytosis (receptor mediated by either clathrin or caveolin), macropinocytosis, phagocytosis or even by passive diffusion [10]. The primary routes of exposure to NMs include the skin, respiratory and GI tract [11]. While studies so far have shown that NMs cannot penetrate into the deep layers via an intact skin, a few studies revealed that minute quantities of NMs can be translocated to secondary organs via the circulation, following pulmonary or gastrointestinal exposure to very high doses of NMs (as reviewed by Krug [12]) although no functional significance has been identified. Nevertheless, there are some studies which also clearly demonstrate that NMs do not translocate to secondary organs [13].

Moreover, the biological effects of NMs appear to be time-dependent, dose-dependent and cell type-dependent [11]. It is known that the interaction, localization and the toxicity of NMs depend largely on their unique physico-chemical properties such as size, aggregation, shape, purity, coating on the surface, composition and solubility [14]. Broadly, NMs less than 100 nm in dimension can enter the cell, while those that are less than 40 nm can enter the nucleus, and those which are smaller than 35 nm can cross the blood–brain barrier [15]. However, in addition to the size of the NM, the cell type, type of NMs used and surface modifications, are other factors that affect uptake. For example, Oh *et al.* [16] showed that poly-ethylene glycolated gold nanoparticles (AuNP) about 2.4 nm in diameter, were found in the nucleus while larger AuNPs of 5.5 nm and 8.2 nm localized in the cytoplasm of Cos-1 cells. Similarly, Huang *et al.* [17] reported that 2 nm and 6 nm AuNPs localized in the cytoplasm of HeLa cells, while 15 nm AuNPs were only present in the cytoplasm of HeLa cells.

Cytotoxicity & genotoxicity

The toxic effect of NMs occurs as a result of their direct or indirect interaction with biological molecules such as lipids, protein and nucleic acids [18]. NMs evoke biological response in cells following physical or chemical interactions that occur during their internalization or uptake [19]. Chemical interactions include production of reactive oxygen species (ROS) [14], peroxidation of lipids [20], altered electron transport [21] while physical interactions include disruption of membrane [22] or alteration of protein folding or aggregation [23,24]. The size, surface functionalization and surface charge of NMs determine their uptake and cellular localization. For example, cationic NPs are more cytotoxic as they are known to disrupt the integrity of negatively charged plasma membrane [25]. Besides, positively charged quantum dots (QD) are internalized to a great extent by cells [26]. In addition, NMs have been found to localize in lysosomes [27] and mitochondria altering the morphology and function of the organelles [28,29].

In both *in vivo* and *in vitro* systems, the interactions of NMs with several proteins (that include serum, plasma proteins and lysosomal proteins following phagocytosis) have been observed resulting in the formation of protein corona surrounding the NMs [30,31]. For example, silica and polystyrene NPs showed preferential adsorption to specific plasma proteins that increased with plasma protein concentrations [32]. Depending on their affinity to NMs as well as to other proteins, the corona can be hard in consistency and composed of proteins that are tightly bound, or soft, containing proteins that are loosely bound [33]. In addition, Brown *et al.* [34] have demonstrated that smaller carbon black NPs (14 nm) interact with cytokine proteins such as TNF alpha and IL-8, resulting in inhibition of cytokine function as well as modifications to their surface reactivity. Furthermore, 45 nm Au and 20 nm titanium dioxide (TiO_2) NPs have been shown to induce changes in the structure of BSA and tubulin proteins [30,35–36].

NMs can enter the nucleus via diffusion or may be transported through the nuclear pore complexes or accidentally, when the cell undergoes mitosis where the nuclear membrane breaks down [37–39]. For instance, exposure of TiO_2 NPs to rats *in vivo* (22 nm) or pulmonary macrophages *in vitro* (0.2 and 0.078 microns) resulted in localization of NPs in nucleus via diffusion [40], while 2.1 nm QDs have been shown to be transported in to the nucleus via nuclear pore complexes in human macrophages [41]. NM-induced genotoxicity may be direct, via intercalation or physical interaction, or indirect via oxidative stress or altering proteins involved in cell division [37,42–43]. TiO_2 and silica NPs have been shown to enter the nucleus and alter cell proliferation by inhibiting DNA replication and transcription processes [24] while QDs have been shown to interact with histone proteins [41].

Increase in ROS levels has been a characteristic phenomenon of NM exposure. It has been previously reported that increased intracellular ROS levels were observed, following exposure of human MRC 5 lung fibroblast or small airway epithelial cells to 20 nm AuNPs [44–46]. Furthermore, 64 nm polystyrene particles has been shown to elicit a greater influx of

neutrophils in rat lungs *in vivo* when compared with 202 and 535 nm sized NPs, suggesting that smaller NPs elicit a greater inflammatory reaction when compared with the larger ones [47]. In addition, the composition of NMs appears to be critical, as NMs with a component of iron can fuel increased generation of hydroxyl radicals [48]. Studies have demonstrated that, cobalt and silica NPs elicit a greater inflammatory response when compared with TiO_2 and zinc oxide NPs while the inflammatory response was absent when nickel oxide or iron oxide NPs were used, suggesting NM-dependent biological toxicity [49,50].

ROS and ROS-induced superoxide anions and hydroxyl radicals can cause damage to DNA [51] or peroxidation of lipids. For example, Fullerne C-60 nanoparticles cause lipid peroxidation in human dermal fibroblasts and the effect was reversible in the presence of ascorbic acid, an antioxidant [52]. In addition, oxidative stress may cause point mutations, DNA adducts, fragmentation of chromosomes promoting DNA damage [53,54] indirectly, by activating intracellular signaling pathways such as MAPK and NF-κB, and triggering inflammation and release of pro-inflammatory cytokines [54]. However, treatment with antioxidants or coating NMs with antioxidant drugs has been shown to rescue NM-induced cellular oxidative stress [55,56]. For example, QDs induced oxidative stress in cells can be prevented by either treating cells or modifying the QDs with antioxidant drugs, such as N-acetylcysteine and lipoic acid [26,55,57]. Damaged DNA is rescued by DNA repair mechanisms in the cell, thereby maintaining genomic stability, failing which carcinogenesis may result [37]. NPs have also been shown to interact and alter the function of DNA repair genes resulting in aberrant DNA repair [46,58]. Exposure of human lung and brain cell lines to silver (Ag) NPs resulted in altered expression of genes involved in DNA damage/repair, such as *Gadd45*, *XRCC1* and *3*, *RPA1*, *RAD51C* and *FEN1*, leading to defects in DNA repair [59].

Furthermore, NMs are known to alter the immune system and cause activation of the complement pathway or promote allergies. For example, lipid-based NPs such as micelles and liposomes, may trigger an innate immune response by activating the complement cascade [60], while carbon nanotubes were shown to promote allergies in Balb/c mice sensitized with ovalbumin [61].

NMs are not always cytotoxic

There are *in vitro* and *in vivo* studies reported in the literature, that has also shown that NMs do not cause overt toxicity. For example, exposure of HepG2 cells or calf thymus DNA to AuNPs *in vitro* did not induce oxidative damage, cell death or generation of free radicals [62]. Similarly, exposure of rainbow trout fish to a diet containing single-walled carbon nanotube or C_{60} fullerenes did not result in any apparent toxicity [63]. Moreover, TiO_2 NPs were found to be not cytotoxic to different aquatic species, while nanosilver and nanocopper were toxic to all species tested, suggesting that sensitivity to NPs depend on species as well as the NP type [64].

Epigenetics & nanotoxicity

While numerous studies have explored the genotoxic potential of ENMs [65], very few studies, so far, have assessed their epigenetic potential. The term 'epigenetics' refers to "*heritable and reversible changes in gene expression without alterations of underlying DNA sequence*" [66]. Gene expression is regulated by three epigenetic mechanisms namely DNA methylation, histone modification and miRNA. Discovered in the 1970s, DNA methylation remains the most widely studied epigenetic mechanism while histone modifications and miRNAs were discovered in 1990s and early 2000 respectively [67,68].

Interestingly, there is some basic information available on the epigenetic mechanisms underlying the toxicity of the bulk materials used for their synthesis. The epigenetic toxicity of heavy metals has been well studied (as reviewed in [69,70]) and several heavy metals such as lead, arsenic, chromium, copper, nickel and cadmium, are known to alter DNA methylation and histone modifications. For example, cadmium used for fabricating cadmium selenide (CdSe) and cadmium telluride (CdTe) QDs, is a known carcinogen that alters DNA methylation. Short-term exposure (1 week) to cadmium has been shown to decrease global DNA methylation, by inhibiting the activity of DNA methylating enzyme, while long-term exposure (10 weeks) results in increased DNA methylation [71]. Similarly, exposure of human lung cells to nickel compounds resulted in decreased acetylation levels of the four core histones, and increased H3K9me and H3K9me2 levels via inhibition of H3K9 demethylase [72].

The environment also contains airborne particles in the nanometer range such as particulate matter (PM, less than 100 nm to 2.5 μm), carbon black particles (10–500 nm), diesel exhaust particles (DEP, 20–130 nm) and cigarette smoke (10–700 nm with average dimension of 150 nm) [73–75]. Although epigenetics-mediated toxicity of ENMs remains the major focus of this review, studies performed on animals and humans exposed to airborne particles in the nanosize range have been included, as these particles have been shown to alter epigenetic mechanisms such as DNA methylation, histone modifications and miRNA expression. The results of such studies are crucial in understanding the epigenetic toxicity of ENMs.

DNA methylation

DNA methylation involves the addition of methyl group to the 5th position of cytosine residue resulting in 5′methylated cytosine [76]. Addition of methyl group occurs on cytosine residues of CpG dinucleotides where a cytosine base is followed by a guanine base [77]. The promoter region of 70% of mammalian genes contain 1000 bp long stretch of CpG sites referred to as CpG islands and are normally unmethylated [78]. Hypermethylated CpG islands inhibit gene expression and are associated with imprinted genes, carcinogenesis and X-chromosome inactivation in females while hypomethylation of CpG islands facilitates gene expression [79]. In mammals, DNA methylation is known to be mediated by three types of DNA methyltransferases (DNMTs), *viz.,* DNMT1, DNMT3A and DNMT3B. While DNMT1 is a maintenance methyltransferase, that duplicates the methylation pattern from the parent strand to the daughter strand following DNA replication, the other two DNMTs are *de novo* methyltransferases that establish new methylation patterns in naked (unmethylated) DNA [80]. DNA methylation prevents the binding of transcription factors to target genes, thereby repressing gene expression either directly or indirectly via methyl CpG binding proteins (MBDs) [81].

In 2008, Choi *et al.* were the first to report that ENMs cause significant epigenetic toxicity [82]. In their study, treatment of human MCF-7breast cancer cells with CdTe QDs (5 μg/ml) decreased DNA methylation in these cells. The epigenetic changes observed in this study were concomitant with changes in expression of specific apoptotic and antioxidant genes. NMs have been shown to affect both global DNA methylation levels, methylation at specific gene promoters as well as enzymes/proteins involved in regulation of DNA methylation such as MBDs and DNMTs. Exposure of human keratinocytes HaCaT cells to silicon dioxide (SiO_2) NPs has been shown to induce global DNA hypomethylation due to reduced expression of DNMTs and MBD2 [83]. Moreover, a recent study by the same group has shown a dose-dependent decrease in the expression of DNA repair gene *PARP-1*, concomitant with increasing levels of DNA methylation in the *PARP-1* promoter in SiO_2 treated HaCaT cells. However, this decrease in *PARP-1* expression could be rescued when DNMT1 was silenced in these cells [83,84], thereby implicating that NMs alter DNA methylation via DNMTs.

The transplacental epigenetic effects of NMs have not been well studied. In a study on pregnant women, hypomethylation of placental DNA was found in women exposed to air pollution early in pregnancy [85]. In another study, no changes in DNA methylation were observed in murine neuronal cells exposed to copper oxide (CuO) NPs *in vitro* [86]. It is not known whether CuO has similar effects on DNA methylation in other cell types/tissues. Exposure of HaCaT cells *in vitro* to TiO_2 NPs impaired the methionine cycle and reduced methionine levels in these cells, suggesting deregulated DNA methylation and also increased oxidative stress by reducing glutathione levels. Although these NPs were found to be localized in the mitochondria, they were not present in the nucleus or other cytoplasmic organelles, but could still alter methylation capacity, possibly by indirect mechanisms [87].

The effects of small particles in the nanoscale range on DNA methylation, have been investigated in humans exposed to airborne fine and ultrafine particles. The expression levels of repetitive elements, such as Alu and long interspersed nucleotide element 1 (LINE-1), were investigated in the leukocytes of elderly patients following short-term or long-term exposure to traffic particles [88,89]. The authors found that the methylation of LINE-1 in leukocyte DNA was decreased in subjects recently exposed to black carbon (a tracer of traffic particles), while there was no association with methylation of Alu elements [73]. However, hypomethylation of both LINE-1 and Alu repetitive elements, were observed in long-term exposure patients [74]. In a similar study, hypermethylation of repetitive elements LINE-1 and Alu were observed in non-smoking Polish workers exposed to PM from coke oven compared with controls [90]. Although the functional significance of these studies remains unclear, these studies signify that DNA methylation can be altered by small particles.

Animal studies on the effects of air pollution on DNA methylation have yielded similar results. It has been reported that C57BL/CBA mice exposed to polluted air that was either filtered (control) or not filtered using HEPA filter, exhibited a 1.6-fold increase in frequency of sperm mutation and increased DNA strand breaks compared with the controls [91]. In addition, these mice contained hypermethylated sperm DNA raising the concern that germ-line mutations can be passed on to the offspring and may cause developmental changes/disease.

Changes in methylation of specific gene promoters have been identified both *in vitro* and *in vivo*, following exposure to ambient PM. Hypomethylation of *iNOS* gene has been observed in adults and children exposed to PM [92,93], while hypermethylation of *Foxp3* locus has been identified in children exposed to air pollution [94]. Similarly, hypermethylation of the *IFNG* promoter and hypomethylation of the *IL-4* promoter of CD4 positive T-cells, were identified in BalB/c mice exposed to DEP and sensitized to a fungal allergen, suggesting that nano-sized particles may promote allergic reactions [95].

Histone modifications

Eukaryotic chromatin is composed of DNA wrapped around histone proteins, thereby facilitating packaging of DNA within the nucleus. Two out of the four core histones (H2A, H2B, H3 and H4) form an octamer, around which 147bp of DNA is wrapped, giving rise to nucleosomes which are fundamental units of chromatin [96]. Adjacent nucleosomes are linked by the linker histone H5 [97]. Gene expression is regulated by post-translational modifications at the N-terminus of the core histones, and about 12 post-translational modifications (such as methylation, acetylation, sumoylation and phosphorylation) that occur in over 60 different residues (that includes lysine, arginine and others) on the histone proteins have so far been identified [67,97]. These post-translational modifications regulate gene expression by altering the packaging of chromatin, thereby modifying the accessibility of DNA to transcription factors, either directly by changing the charge on histone proteins, or indirectly by employing proteins that bind to specific modifications [67]. Depending on the post translation modifications to histone and the presence or absence of DNA methylation, the level of chromatin condensation varies and chromatin exist as euchromatin (less condensed and contains transcriptionally active genes) or heterochromatin (well condensed and present in centromere, telomere regions of chromosomes) [98].

Histone acetylation and methylation are mediated by enzymes that add or remove specific groups to the histone proteins. Histone acetylation is mediated by two opposing groups of enzymes, namely, histone acetyltransferases which add acetyl groups, and histone deacetylases (HDACs) that remove them. Histone methylation is facilitated by histone methyltransferases that add methyl groups to histone residues resulting in activation or repression of transcription that can be reversed by histone demethylases [67].

NP induced changes in histone modifications were first reported in the treatment of human MCF-7 breast cancer cells with CdTe QDs (5 μg/ml) [82]. The authors identified chromatin reorganization, and dose-dependent decrease in acetylation of histone H3. Furthermore, their study indicated that QD-induced hypoacetylation could be reversed in the presence of trichostatinA, a HDAC inhibitor (HDACi), suggesting that histone modifying enzymes may be involved in NP-induced modifications to histone.

We have observed chromatin condensation and reorganization in fetal lung fibroblasts treated with AuNPs [44] while exposure of small airway epithelial cells to AuNPs resulted in decreased expression of a repressor mark, histone H3 lysine27 trimethylation (H3K27me3), when compared with the control cells (Figure 1). Similarly, CdTe QDs have been shown to induce chromatin condensation and apoptosis in rat PC12 cells upon exposure [56]. Furthermore, 3.4 nm sized negatively charged thioglycolic acid capped CdTe QDs, have been shown to bind preferentially to positively charged histone proteins *in vitro* and not to nucleic acids, resulting in considerable alterations to the size and charge of the NPs [99]. The size of the NPs increased from 3.4 to 150 nm and 225 nm in the presence of lower and higher concentrations of core histone, respectively. In addition, this preferential binding indicates that the charge of NMs may be crucial in determining their nuclear localization and interaction with the positively charged histone protein. It has also been shown that AuNPs bind to thiol groups on HDAC 8 in the presence of NaCl, thereby inhibiting its function [100]. This property of NPs offers tremendous therapeutic potential in cancer research. HDACi delivering prodrugs have been found to increase gene expression by two to fourfolds due to increased acetylation of core histones [101].

Oxidative stress has been shown to increase the levels of acetylated histone H4 [102]. Therefore, changes in histone acetylation observed upon treatment with NMs may be indirect since NMs induce oxidative stress in cells. Changes in histone modifications have also been observed upon exposure to PM and DEP. Treatment of A549 cells with PM resulted in increased release of IL-8, and increased acetylated histone H4

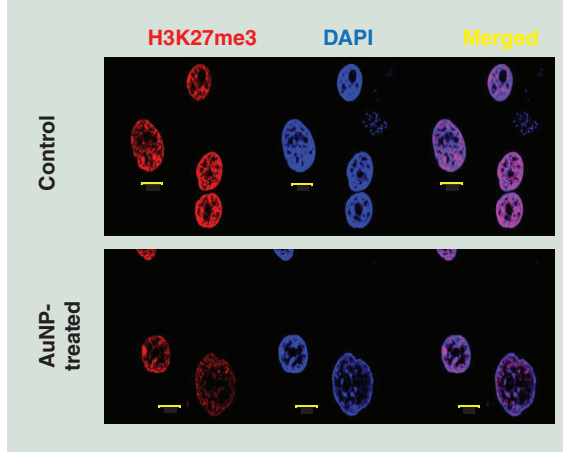

Figure 1. Expression of H3K27me3 in control and gold nanoparticle-treated small airway epithelial cells.
Confocal microscopy images of small airway epithelial cells stained with antitrimethylated histone H3 lysine 27 (H3K27me3, stained red by Cy3) in control and AuNP-treated cells. The nucleus is counterstained with DAPI. AuNP-treated cells were exposed to 20 nm size AuNPs for 72 h (Scale bar: 100 μm).
AuNP: Gold nanoparticle; DAPI: 4′,6-Diamidino-2-phenylindole dihydrochloride.

in the *IL-8* gene promoter [103]. Furthermore, the increase in IL-8 was enhanced upon treatment with HDACi with a corresponding decrease in the expression of HDAC2 in the nucleus. Similarly, exposure of bronchial epithelial cells to DEP increased the expression of *Cox-2* via increasing acetylation of histone H4, by recruiting histone acetyltransferase in the *Cox-2* promoter, as well as, degradation of HDAC1 [104]. In another study, time-dependent and metal type-dependent changes in histone modifications were identified in blood leukocytes of steel workers exposed to PM containing metals such as aluminum, manganese, nickel, zinc, arsenic, lead and iron [105]. The study revealed a significant increase in H3K4me2 and H3K9ac that were associated with years of employment, and exposure to nickel, arsenic or iron which resulted in increased H3K4me2 levels but not H3K9ac levels. Furthermore, cumulative exposures to arsenic and nickel air levels showed a positive correlation to H3K4me2 and H3K9ac, suggesting that histone modifications depended on duration of exposure and the metal type. These studies suggest that PM and DEP can interact with histone and histone modifying enzymes similar to ENMs.

Expression of miRNA

miRNAs are small, single stranded, noncoding RNA molecules that act as translational repressors. Initially miRNAs were identified in *Caenorhabditis elegans*, but they have now been shown to be expressed ubiquitously by all species [106,107]. miRNAs have diverse functions in cells and it is estimated that about a third of the mammalian genes are regulated by miRNAs [108]. At the 5′ end of the mature miRNAs, the nucleotides 2–8 are referred to as the 'seed region' that determine the mRNA target. Based on complementarity between the miRNA seed region and mRNA target, the mRNA may be degraded if there is complete complementarity (mostly observed in plants), or protein translation or mRNA deadenylation may be inhibited when there is partial complementarity as mostly seen in mammalian cells [109,110]. Based on seed complementarity, each miRNA is predicted to target numerous mRNAs while in turn each mRNA can be a target of several miRNAs [111].

AuNPs have been shown to alter miRNA expression both *in vitro* and *in vivo*. We observed that exposure of fetal lung fibroblasts to AuNPs, altered the expression of 19 genes including *PROS-1* and miR-155 [44]. miR-155 is known to regulate various cellular pathways that are critical for inflammation, carcinogenesis and cardiovascular diseases [112] while the *PROS-1* gene encodes for a glycoprotein namely, Protein S, that negatively regulates thrombus formation [113]. Furthermore, the *PROS-1* gene which was found to be highly expressed by human lung tissues compared with other tissues, may be a miR-155 target, suggesting that AuNPs induced *PROS-1* expression could be regulated by miR-155. In another study, the expression of several miRNAs was altered in fetal lung and liver following maternal exposure to AuNPs. Furthermore, the expression of two miRNAs, miR-183 and Let-7a were found to be increased in both fetal liver and lungs [114], although their targets and function remain unknown.

In a study by Li *et al.*, mouse 3T3 fibroblasts treated with three different types of NMs (iron oxide NPs, CdTe NPs and multiwalled carbon nanotubes) were observed to have co-regulated miRNAs (miR-34s, miR-21 and miR-29a) and common deregulated miRNA targets, suggesting comparable effects of NMs on miRNA expression [115,116]. Pathway analysis performed recently by the same authors revealed apoptosis like-cell death as the main pathway predicted by miRNAs deregulated upon exposure of 3T3 cells to CdTe QDs *in vitro* [117], which is consistent with previous studies where apoptotic cell death was reported upon CdTe QD exposure [57,118]. AgNPs have been demonstrated to alter the expression of several miRNAs that regulate bone mineralization in cultured osteoblasts [119]. In addition, the deregulated miRNAs were found to target genes involved in several biological pathways including stress, cell signaling, synthesis and degradation of proteins. Exposure of mice to TiO_2 nanoparticles via whole body inhalation resulted in a 60-fold upregulation of miR-135b, concomitant with altered expression of genes involved in inflammation and immune response [120]. In a study by the same group, exposure of mice to carbon black particles or cigarette smoke resulted in upregulation of miR-135b [121,122], suggesting common miRNA targets for nano-sized materials and significant roles for miR-135b in pulmonary toxicity. Changes in miRNA expression post NM exposure have been rarely explored in *in vivo* models. A study by Chew *et al.* identified miRNA changes in rat blood taken 1 week and 2 month after a single intravenous injection of AuNPs [123]. They found 23 and 45 dysregulated miRNAs in rats after 1 week and 2 months post AuNP exposure, respectively. They also identified miRNAs that were expressed in common for both AuNP exposed groups and explored the potential of using miRNA expression as blood biomarkers.

In a recent study, a combination of gene expression microarray and miRNA microarray has revealed that miRNAs were involved in regulating differential sensitivity seen in Jurkat T cells exposed to AgNPs or Ag ions [124]. This study showed that expression of 63 miRNAs and 15 mRNAs were altered upon exposure

to AgNPs, and 32 miRNAs and 4mRNAs to Ag ions, respectively. Furthermore, the AgNPs-induced expression of *MT1F* and *TRIB3* genes were correlated negatively with the expression of miR-219–5p and Ag ion-induced expression of *ENDOGL1* with miR-654–3p, suggesting that these miRNAs were involved in regulating the gene expression. In addition, pathway analysis revealed that miR-219–5p and its gene targets, *MT1F* and *TRIB3*, were involved in several cellular processes such as oxidative stress, apoptosis and cell cycle, which were associated with AgNP-induced toxicity in Jurkat T cells. Whereas, pathway analysis of miR-654–3p and its target gene *ENDOGL1* showed networks suggesting that miRNAs may be critical determinants of NP toxicity. Such studies that utilize high-throughput techniques, such as global gene microarray combined with pathway analysis, would help to reveal the functional significance associated with the observed epigenetic changes, thereby identifying molecular mechanisms underlying NM-induced toxicity.

miRNA expression is known to be modulated upon exposure to cigarette smoke, PM and DEP. Altered expression of more than 60% of miRNAs has been reported in lung cells following exposure to DEP [125]. In a very recent study, cigarette smoke was reported to alter the expression of several miRNAs and DICER (which is an enzyme involved in miRNA biosynthesis) in alveolar macrophages [126]. In another study, specific miRNAs (miR-222 and miR-21) were found to be upregulated in the blood leukocytes of steel plant workers exposed to PM [127]. In addition, the same authors found that increased expression of miRNA was positively correlated with exposure to a specific metal (miR-222 and lead) and also with increased DNA damage (miR-21 and blood-8-hydroxyl guanine).

Methods for detecting epigenetic changes

Numerous genome wide or gene-specific techniques have been developed for the analysis of epigenetic mechanisms. The bisulphite conversion of DNA continues to be the gold standard, offering resolutions for analyzing methylated DNA, up to a single methylated cytosine. Following bisulphite conversion, the DNA can be amplified by PCR or cloned into plasmid and sequenced. Depending on the applications, quantity of DNA and sources of the samples, bisulphite sequencing of DNA can be followed by methylation specific PCR (MSP), combined bisulphite restriction analysis, sensitive melting analysis after real time MSP or methylation-sensitive high resolution melting. To examine genome wide DNA methylation levels, methylated DNA immunoprecipitation using antibodies against 5′ methylated cytosine remains the widely used technique [128]

Chromatin immunoprecipitation (ChIP) offers identification of DNA–protein interactions as well as post translational modifications to histone protein. ChIP can be modified based on the application, namely as native ChIP or micro-ChIP and offer several advantages over the conventional ChIP technique. Following ChIP, the enriched DNA can be analyzed by real time PCR, to identify specific post translational modifications on a gene, or combined with high-throughput techniques such as DNA sequencing (ChIP Seq) or DNA microarray (ChIP on chip) to identify genome wide protein binding sites. Recently, new techniques have been developed to identify the crosstalk between DNA methylation and histone modifications (such as ChIP with bisulphite methylation sequencing) that will help in understanding the dynamic epigenetic mechanisms and their crosstalks better [128].

Global miRNA expression is analyzed using microarray or deep sequencing, while specific miRNAs can be identified using real time PCR, northern blot or *in situ* hybridization. In order to study the function of miRNAs, the genes or pathways targeted by a specific miRNA should first be known. Several databases such as Targetscan, miRanda, miRWalk are freely available to predict miRNA–gene interactions. Following target prediction, validation of miRNA–gene interactions can be done by loss or gain of function approach using miRNA mimics/inhibitors or by 3′UTR analysis [129].

Conclusion & future perspective

The epigenetic mechanisms of toxicity induced by ENMs remain largely unexplored. Of the available studies, several groups have identified changes in miRNA expression and DNA methylation following NM exposure, but there is still a lack of information about modifications to histone proteins. NM-induced changes in gene expression have been identified by various studies, and it is likely that NMs could alter gene expression via miRNAs given that miRNAs fine tune gene expression. Therefore, it would be worthwhile to further explore the role of miRNAs in NM-induced toxicity. Among histone modifications, only histone methylation and acetylation have been found to be modified by NMs while other post-translational modifications to histone proteins, such as sumoylation and phosphorylation, have not been studied. Most researchers have largely focused on only one epigenetic mechanism but the interplay between the different epigenetic mechanisms needs to be investigated to understand the mechanisms by which nanoparticles induce toxicity. Similarly, the functional significance of the identified epigenetic changes is still under investigation, while the

Table 1. Studies on epigenetic mechanisms altered by engineered nanomaterials.

NM	Size	Dose	Duration	Cell type	Type of epigenetic damage	Methods used for analysis	Ref.
DNA methylation							
CdTe QDs	-	5 μg/ml	4 h/24 h	MCF7 breast cancer cells	Reduced global DNA methylation levels	Immunocytochemistry using anti 5-methyl cytosine antibody	[82]
SiO_2	15 nm	2.5, 5, 10 μg/ml	24 h	HaCaT epidermal keratinocytes	Decreased global DNA methylation that increased with dose	Immunocytochemistry and flow cytometry using anti 5-methyl cytosine antibody and HPCE assay	[83]
					Decreased expression of DNMT 1 and 3a and MBD2 that increased with dose	Real time RT-PCR and western blot	
	1–5 μm	10 μg/ml	24 h	HaCaT epidermal keratinocytes	Decreased global DNA methylation	HPCE assay	
					Decreased expression of DNMT 3a mRNA	Real time RT-PCR	
	15 nm	2.5, 5, 10 μg/ml	24 h	HaCaT epidermal keratinocyte	Dose-dependent decrease in PARP-1 gene expression by increasing DNA methylation (CpG specific hypomethyaltion) at PARP-1 promoter	Real time RT-PCR and western blot Methylation specific PCR and bisulphite sequencing	[84]
CuO	70–100 nm	6.25–400 mg/l	24 h	Neuro2A mouse neuroblastoma cell line	No change in DNA methylation	Quantification of 5-methyl deoxy cytosine (m^5dC)/cytosine (dC) ratio by HPLC	[86]
TiO_2	10–100 nm (identified)	5, 50, 100 μg/ml	24 h	HaCaT epidermal keratinocyte	Impairment of methionine cycle and reduced methionine levels suggesting deregulated DNA methylation	Gas chromatrography/ mass spectrometry or liquid chromatography/mass spectrometry	[87]
Histone modification							
CdTe QDs	-	5 μg/ml	4 h/24 h	MCF7 breast cancer cells	Chromatin condensation	Live cell imaging of nuclei, electron microscopy, and 3D nuclei reconstruction	[82]
		0.5–10 μg/ml			Decreased acetylated histone H3 (dose dependent)	Immunofluorescence and Western blot	
AuNPs	20 nm	1 nM	48 h	MRC 5 fetal lung fibroblasts	Chromatin condensation and reorganization	TEM and immunofluorescence	[44]

AgNP: Silver nanoparticle; AuNP: Gold nanoparticle; CdTe: Cadmium telluride; CuO: Copper oxide; Fe_2O_3: Iron oxide; HPCE: High performance capillary electrophoresis; HPLC: High-performance liquid chromatogrpahy; MWCNT: Multi walled carbon nanotube; NM: Nanomaterial; NP:Nanoparticle; QD: Quantum dots; SiO_2: Silicon dioxide; SOLiD: Sequencing by oligonucleotide ligation and detection; TEM: Transmission electron microscope; TiO_2: Titanium dioxide.

Table 1. Studies on epigenetic mechanisms altered by engineered nanomaterials (cont.).

NM	Size	Dose	Duration	Cell type	Type of epigenetic damage	Methods used for analysis	Ref.
CdTe	2.2 nm	37.5 μg/ml	24 h	Rat Pheochromocytoma PC12 cells	Chromatin condensation	Immunofluorescence	[56]
CdTe	3.4 nm	1×10^{-6} M	30min	Phagocytic THP1 cells	Preferentially bind to positively charged histone proteins and not nucleic acids resulting in considerable alterations to the size and charge of the NPs	Modified dot blot technique	[99]
AuNPs				Cell-free medium	Bind and inhibit histone deacetylase 8	Spectrophotometer (Absorbance)	[100]
miRNA expression							
AuNPs	20 nm	1 nM	48 h	MRC 5 fetal lung fibroblasts	Differential expression of 19 genes including upregulation of miR-155;	Gene expression microarray	[44]
					miR-155 may regulate *PROS-1* gene	miR functional studies (miR inhibitors and reporter assay)	
AuNPs	40 or 100 nm	3.3 mg/kg	Samples harvested on gestational day 18	Intraperitoneal injection in pregnant swiss albino mice (strain H) on gestational days 10, 12,14 and 17	28 miRNAs and five miRNAs were deregulated in fetal lung and liver respectively (only observed in 100 nm dose) suggesting transplacental and clastogenic effects Let7a and miR-183 were upregulated in both organs in (only in 100 nm dose)	miRNA expression profiling by microarray	[114]
Fe_2O_3, CdTe and MWCNT	-	Fe_2O_3 and MWCNT: 100 μg/ml CdTe: 30 μg/ml	24 h	Mouse NIH/3T3 fibroblasts	Differential expression of miRNAs (Fe_2O_3: 16.13%, MWCNT: 9.05% and CdTe: 17.75%) upon treatment with three different NPs. Co-regulated miRNA expression in any two NP treatment and miR-34a, miR-21 and miR-29a were common deregulated miRNAs in all three NPs tested	miRNA expression profiling by SOLiD sequencing	[115]
CdTe QDs		15, 30 and 45 μg/ml	12 h (15 μg/ml) 24 h (30, 45 μg/ml)	Mouse NIH/3T3 fibroblasts	miRNA processing was altered since transcript levels of pri-miRNA affected	miRNA expression profiling by SOLiD sequencing and Z test	[116]

AgNP: Silver nanoparticle; AuNP: Gold nanoparticle; CdTe: Cadmium telluride; CuO: Copper oxide; Fe_2O_3: Iron oxide; HPCE: High performance capillary electrophoresis; HPLC: High-performance liquid chromatogrpahy; MWCNT: Multi walled carbon nanotube; NM: Nanomaterial; NP:Nanoparticle; QD: Quantum dots; SiO_2: Silicon dioxide; SOLiD: Sequencing by oligonucleotide ligation and detection; TEM: Transmission electron microscope; TiO_2: Titanium dioxide.

Table 1. Studies on epigenetic mechanisms altered by engineered nanomaterials (cont.).

NM	Size	Dose	Duration	Cell type	Type of epigenetic damage	Methods used for analysis	Ref.
					Expression of 20 miRNAs and four miRNAs were dose dependent and time dependent respectively	Real time-RT PCR	
CdTe QDs		30 μg/ml	24 h	Mouse NIH/3T3 fibroblasts	Apoptosis-like cell death was the top pathway predicted to be targeted by the deregulated miRNAs	Mathematical model	[117]
AgNP	23 nm (±2 nm)	20 μg/ml	6 days	M3CT3 bone cells	miRNA expression was analyzed 6, 15 and 24 days post exposure to AgNPs; Expression of few miRNAs that target genes involved in bone-mineralization were altered in cells 6, 15 and 24 days post exposure	miRNA PCR array miRNA–mRNA target prediction	[119]
TiO_2	4–200 nm	42.4 ± 2.9 mg/m^3	Whole body inhalation 1 h/day for 11 days and samples collected 5 days post exposure	Female C57BL/6BomTac mice	Expression of several miRNAs that target genes involved in inflammation and immune response in the lung were altered	miRNA expression profiling by microarray Gene expression analysis using cDNA microarrays and pathway specific PCR arrays	[120]
					Expression of miR-135b was increased 60-folds suggesting critical roles in pulmonary toxicity	Real time-RT PCR	
AuNPs	20 nm	15.1 mg/ml	Single intra venous injection	Male wistar rats Sacrificed 1 week or 2 months post exposure	23 miRNAs and 45 miRNAs were deregulated 1 week and 2 months post exposure and 21 miRNAs were common in both groups implying that miRNAs could be biomarkers of acute or long-term exposure	miRNA expression profiling by microarray	[123]
AgNPs	<100 nm	0.2 mg/l	24 h	Jurkat T cells	63 miRNAs and 15 mRNAs were altered	Gene expression and miRNA expression profiling by microarray	[124]
					miR-219–5p was predicted to target *MT1F* and *TRIB3* genes that were involved in several cellular processes associated with AgNP-induced toxicity	miRNA–mRNA target identified by pathway analysis	

AgNP: Silver nanoparticle; AuNP: Gold nanoparticle; CdTe: Cadmium telluride; CuO: Copper oxide; Fe_2O_3: Iron oxide; HPCE: High performance capillary electrophoresis; HPLC: High-performance liquid chromatogrpahy; MWCNT: Multi walled carbon nanotube; NM: Nanomaterial; NP:Nanoparticle; QD: Quantum dots; SiO_2: Silicon dioxide; SOLiD: Sequencing by oligonucleotide ligation and detection; TEM: Transmission electron microscope; TiO_2: Titanium dioxide.

long-term consequences and trans-generational effects of the altered epigenetic mechanisms are not known.

Thus far, the studies suggest a relation between dose, size and epigenetic effect (Table 1). Both global and site specific DNA methylation appears to be dose-dependent in SiO_2 treated cells [83,84]. Furthermore, Histone H3 hypoacetylation appears to be dose dependent, and detectable at even low concentrations of exposure to QDs (<5 µg/ml) although no apparent changes in cell morphology/cell death were evident at that concentration [82]. However, as in the case of miRNAs, both size dependent and dose-dependent alterations have been reported. For example, maternal exposure to larger AuNPs (100 nm) and not smaller (50 nm), altered the expression of several genes and miRNAs in the fetal liver and lungs [114], while CdTe induced dose-dependent alterations in miRNA expression [116]. Moreover, the same miRNAs were altered following treatment with three different NMs at dissimilar concentrations (Fe_2O_3 and MWCNT: 100 µg/ml, CdTe: 30 µg/ml), suggesting that these miRNAs may regulate similar genes/pathways associated with NM-induced toxicity [115]. In addition to dose dependency, the expression of miRNA appears to be time dependent as well, as seen in CdTe treated cells [116]. Finally, Chew *et al.* [123] identified that the same miRNAs were altered in the blood of AuNP-treated rats at 1 week and 2 months post-exposure, suggesting that epigenetic changes may be persistent.

The potential for artefacts to bias results of nanotoxicity testing may occur at any stage of NM research such as purchase/synthesis of NM, storage, sample type and assay used (as reviewed by Petersen *et al.* [130]). For example, analytical assays (MTS, MTT, LDH and alamar blue) are used mainly to estimate NM toxicity. These analytical assays rely on changes in optical density (absorbance or fluorescence) as endpoints to determine biological effect of NMs, but contain reagents such as dyes or proteins that may interact with NMs,

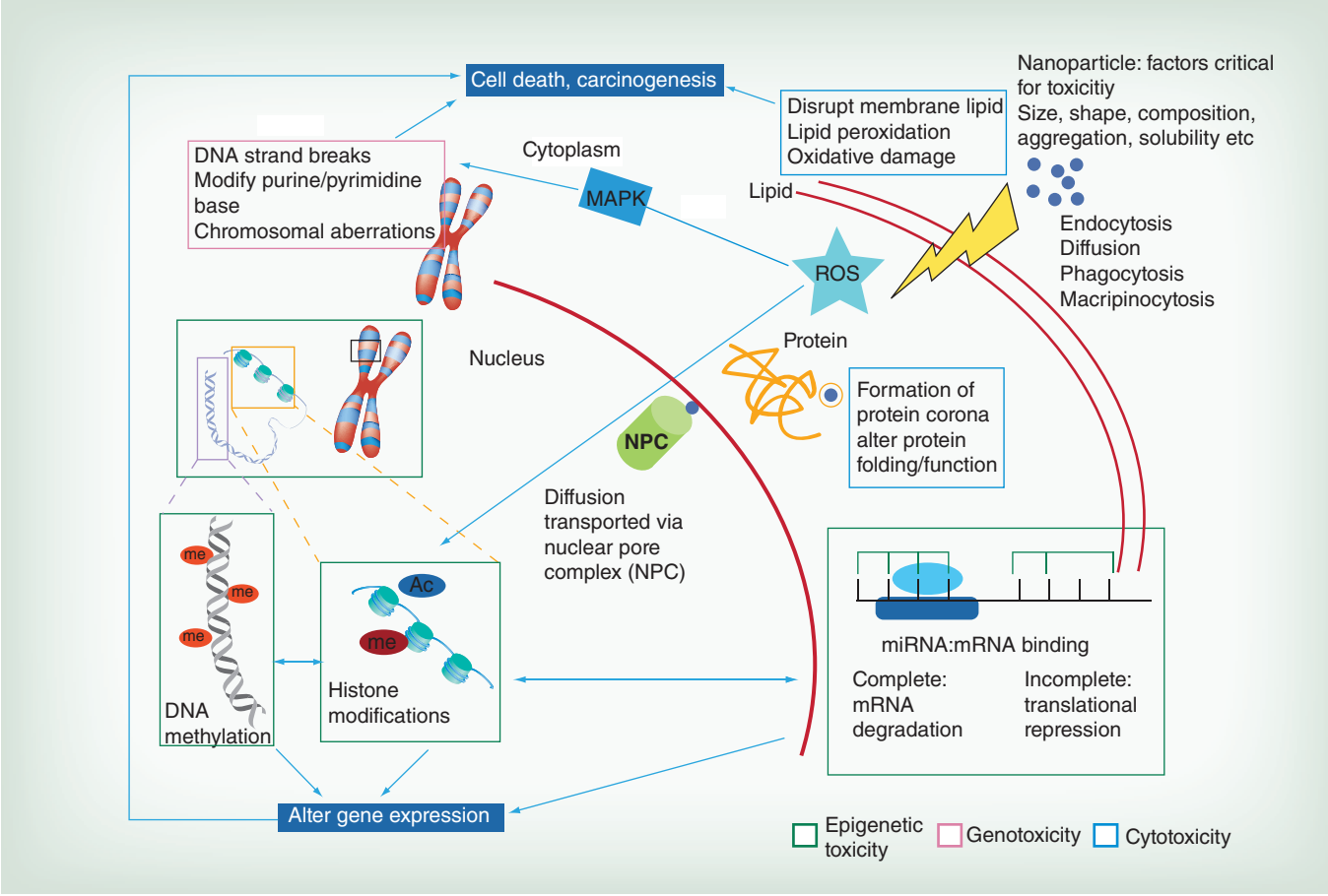

Figure 2. Nanomaterial: interaction and toxicity. Nanomaterials interact with lipids, protein and nucleic acids and their structure and function resulting in genotoxicity, cytotoxicity as well as epigenetic toxicity. The effects of nanomaterial-induced toxicity appear to be interdependent. Epigenetic toxicity is due to altered DNA methylation and/or histone modifications and/or miRNA expression and function, and these epigenetic mechanisms can be driven by one another.
Ac: Acetylated; me: Methylated; MAPK: Mitogen activated protein kinase; NPC: Nuclear pore complex; ROS: Reactive oxygen species.

resulting in artefacts and inconsistencies in estimation of NM toxicity (as reviewed by Ong *et al.*, [131]). Hence, the experimental design and interpretation of data should be handled with caution. As far as epigenetic mechanisms are concerned, the changes seen may not be driven by NMs directly but rather due to NM-mediated stress. Epigenetic changes observed may not be specific to any NM. Future studies should analyze and compare the different epigenetic mechanisms in cells exposed to various NMs. However, assays to quantitate epigenetic mechanisms should be selected carefully along with the appropriate controls, as with any experiment involving NMs.

Several questions and gaps in literature need to be addressed before the 'safe' use/exposure to NMs. Figure 2 summarizes the various NM-mediated toxicity based on information available in the literature and suggests possible links for future studies. While numerous unique properties of NMs such as size, shape, composition, coating and dose are critical for their genotoxicity, researchers have yet to explore whether the same parameters affect epigenetic toxicity. With studies so far suggesting direct and indirect effects of NMs on DNA methylation, histone modifications and miRNA, future studies could unveil the complexity and interplay between them. In addition, the findings from *in vitro* and *in vivo* studies of the epigenetic effects of NMs needs to be correlated.

Currently, different techniques are employed to assess the NM-induced epigenetic toxicity and therefore a standard guideline is required. Similarly, the results of a single study wherein a NM does alter epigenetic mechanisms in a certain cell type cannot be applied across different cell types as a general rule of thumb. Extensive testing across different cell types is therefore necessary before deeming a NM epigenetically 'safe'. High-throughput techniques (such as miRNA microarray, DNA methylation array and ChIP) combined with pathway analysis will help identify biomarkers or early responses upon exposure to NMs that will hold great therapeutic and commercial potential in the current situation.

Furthermore, data obtained from epigenetic toxicity of nano-sized particles will help provide insights into the biology of ENMs. It would seem that with the rapidly growing use of NMs in day to day items, identification of epigenetic signatures of the different NMs could become a need of the hour.

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Executive summary

Nanmaterial-induced toxicity

- Interaction, localization and the toxicity of nanomaterials (NMs) depend greatly on factors such as size, aggregation, shape, purity, coating on the surface, composition and solubility.
- Toxic effect of NMs is time dependent, dose dependent and cell type dependent.
- Engineered nanomaterials and nano-sized materials induce cytotoxicity, genotoxicity and epigenetic toxicity.

Cytotoxicity & genotoxicity

- The toxic effect of NMs occurs as a result of their direct or indirect interactions with biological molecules such as lipids, protein and nucleic acids.
- NMs can cause peroxidation of lipids resulting in increased permeability to ions and eventually cell death due to loss of flexibility of the phospholipid bilayer.
- Some NMs have been shown to modify the molecular conformation of proteins, thereby altering its function.
- NMs may induce genotoxicity that can be direct, via intercalation or physical interaction with DNA, or indirect via oxidative stress or altering proteins involved in cell division.

Epigenetic toxicity

- NMs have the potential to affect both global DNA methylation levels or methylation at specific gene promoters as well as enzymes/proteins involved in regulation of DNA methylation such as methyl CpG binding proteins and DNA methyl transferases..
- The ionic charge of NMs appears to be critical in determining its interaction with the positively charged histone protein. NMs may alter chromatin organization by inducing post translational modifications (methylation or acetylation) to histone proteins, or by altering the function of histone modifying enzymes such as HDAC.
- Some NMs have been identified to alter the expression and function of miRNAs that regulate genes involved in vital cellular mechanisms.

Future perspective

- Identification of epigenetic signatures of the different NMs is crucial.
- Interplay between the different epigenetic mechanisms need to be investigated.
- Long-term consequences and trans-generational effects of the altered epigenetic mechanisms are unknown.

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