Advanced Review

Metal-based nanoparticles and their toxicity assessment

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Nanoparticles (NPs) can potentially cause adverse effects on organ, tissue, cellular, subcellular, and protein levels due to their unusual physicochemical properties (e.g., small size, high surface area to volume ratio, chemical composition, crystallinity, electronic properties, surface structure reactivity and functional groups, inorganic or organic coatings, solubility, shape, and aggregation behavior). Metal NPs, in particular, have received increasing interest due to their widespread medical, consumer, industrial, and military applications. However, as particle size decreases, some metal-based NPs are showing increased toxicity, even if the same material is relatively inert in its bulk form (e.g., Ag, Au, and Cu). NPs also interact with proteins and enzymes within mammalian cells and they can interfere with the antioxidant defense mechanism leading to reactive oxygen species generation, the initiation of an inflammatory response and perturbation and destruction of the mitochondria causing apoptosis or necrosis. As a result, there are many challenges to overcome before we can determine if the benefits outweigh the risks associated with NPs. *WIREs Nanomed Nanobiotechnol* 2010 2 544–568

Nanoparticles (NPs) can potentially cause adverse effects on organ, tissue, cellular, subcellular, and protein levels due to their unusual physicochemical properties. Metal NPs have received increasing interest in many fields.

The Diverse Applications of Metal Nanoparticles

The revolutionary potential of nanoparticles continues to intrigue scientists, medical professionals, and consumers alike, with novel breakthroughs resulting in an anticipated one trillion dollar industry by 2015.[1–3] In this review, NPs, with lengths of 1–100 nm, will be discussed with special reference to their unique physicochemical properties that are not present in conventional bulk materials. As a result, NPs are not merely small crystals, but an intermediate state of matter somewhere between bulk and molecular materials. Independent of the very small size of NPs, several parameters play a dominant role in their enhanced magnetic, electrical, optical, mechanical, and structural properties. Many of these characteristics have potential implications in NP toxicity, such as elemental composition, charge, shape, crystallinity, surface area, solubility, and surface chemistry/derivatization.[4–12]

The unprecedented freedom to design and modify NPs to accomplish very specific tasks is currently being realized. For example, NPs are being designed with chemically modifiable surfaces to attach a variety of ligands to improve biosensors, imaging techniques, delivery vehicles, and other useful biological tools. The wide spread use of gold nanoparticles (Au NPs) in biological applications has been due to their simple synthesis methods,[13] ease of surface modification with peptides, DNA and antibodies,[14–16] and unique physicochemical properties such as excellent absorbance and scattering of light. On the basis of these properties, Au NPs have important applications for biological diagnostics,[17] cell labeling,[18] targeted drug delivery,[19] medical imaging,[20] cancer therapy,[21–24] and biological sensors.[25] Furthermore, aluminum nanoparticles (Al NPs) have been proposed as drug delivery systems, specifically by encapsulating drugs that are nonionic

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or not water soluble, with aluminum–magnesium hybrids to increase solubility, thus avoiding clearance mechanisms and allowing for site-specific targeting of drugs to cells.[26]

Other areas of biological research and industry have seen a tremendous increase in the production of NPs used as markers in biological imaging such as iron oxide (Fe_2O_3)[27,28] or noble metal plasmon-resonant particles (i.e., Au, Ag, Pt, and Pd). The latter NPs produce an optical signal upon the excitation of surface plasmon resonances, which are collective oscillations of free electrons at the surface of metals.[29–31] These oscillations give rise to the intense colors of solutions of plasmon-resonant NPs, such as silver and other metals/metal-oxides, which have recently been examined for agglomeration, uptake, and interaction in a variety of live cells with a high illumination system.[30]

Silicon dioxide or silica (SiO_2) NPs have great practical importance in industrial applications, such as the fabrication of electric and thermal insulators, media for coating processes, adsorbents, molecular sieves, and filler materials.[32] They are also widely used in biomedical applications as catalyst supports, drug carriers,[33] and gene delivery.[34] Colloidal silica crystals with periodicity within the optical wavelength scale also have a photonic band gap, which makes them well suited for electronic applications ranging from microwave to optical devices.[35] The significance of silica-ordered particle arrays lies in the fact that it is possible to induce wavelength coalescence with the close-packed structure. These particle arrays can diffract light in the UV, visible, and near infrared regions in a manner analogous to X-ray diffraction from ordinary mineral crystals.[35,36] The use of silica in most large-scale electronic devices also finds great potential for use in nanoscale devices. However, nano-sized SiO_2 can readily interact with biomolecules on the cell surface and within the cell often in ways that do not alter the behavior and biochemical properties of those molecules.[37] Recently, investigators have developed methods for chemically modifying lithographically etched silicon nanostructures, enabling attachment to a broad range of molecules. This is the first step in creating versatile chip-based biosensors.[38] Silicon-based arrays made of antibody-conjugated nanowires coupled with transistors have also been multiplexed to simultaneously detect single copies of multiple viruses.[39]

Many metal-based NPs have been heavily researched as candidates for novel antimicrobial (i.e., antiviral, antibacterial, antifouling, and antifungal) applications as biocides, antibiotic treatment alternatives, and nanocomposite coatings.[40–44] Silver nanoparticles (Ag NPs), in particular, are currently being added to many common household products such as bedding, washers, water purification systems, tooth paste, shampoo, fabrics, deodorants, filters, paints, kitchen utensils, toys, and humidifiers to impart antimicrobial properties.[40,45–49] The widespread medical use of Ag NPs as additives can be demonstrated by a plethora of products such as bandages, catheters, and other materials to prevent infection, particularly during the healing of wounds and burns.[50–52] Additionally, Ag NPs are a broad spectrum antimicrobial agent against >650 different types of disease-causing organisms, including viruses.[53–56] The mechanism by which Ag NPs prove to be effective antimicrobial agents is due to their ability to bind to proteins and interfere with bacterial and viral processes.[57,58] Furthermore, Ag can bind to sulfur-based groups such as thiols in mammalian cells.[50] The ability to tailor Ag NPs for specific functions through surface engineering lends itself to a greater variety of products for wound healing[59,60] and the development of novel cancer therapies.[61,62] The most common health effect associated with prolonged dermal exposure to Ag is argyria, a permanent bluish-gray discoloration of the skin.[63]

Furthermore, metal NPs composed of titanium dioxide (TiO_2), copper (Cu), zinc (Zn), Al, and Ag NPs are also receiving considerable attention as additives in consumer and industrial products. TiO_2 is used in cosmetics, filters that exhibit strong germicidal properties and remove odors, and in conjunction with Ag as an antimicrobial agent.[64] Moreover, due to its photocatalytic activity, TiO_2 has been used in waste water treatment.[65] Additionally, surfaces can be made resistant to abrasion with the addition of TiO_2 and Al_2O_3 coatings.[66] However, the dual role of TiO_2 and ZnO to protect the skin from sun damage or become photo-activated to kill surface bacteria (i.e., self cleaning surfaces) raises some concern about confounding bio-effects. Nano-sized Ag (~50 nm in diameter) has been added to the ink of ink jet printers, increasing the ability to print on difficult surfaces such as glass.[67,68]

Copper nanoparticles (Cu NPs) are finding use in a variety of industrial applications as fillers to increase conductivity, improve wear resistance and ductility, reduce friction, and act as catalysts on activated carbons to reduce levels of nitrate in water.[69–72] Similar to Ag NPs, Cu NPs have been shown to inhibit the growth of bacteria such as *Escherichia coli* and *Bacillus subtilis*.[48] The proposed mechanism by which Cu NPs act as effective antibacterial agent against these species is due to interactions with SH

groups leading to protein denaturation.[73] Copper also displays a dual capacity to act as a required cofactor and biocatalyst with a critical balance for proper intracellular metal homeostasis and metabolism and has been implicated in disease conditions.[74,75] For this reason, Cu NPs are undergoing heavy scrutiny to understand potential links between applications, exposure, and disease.

The Far-reaching Implications of Metal Nanoparticles

Due to tremendous advances for the utility of metal-based NPs, there is a great amount of data that has been published on NP properties and toxicity. In this regard, it is extremely difficult to provide a completely comprehensive review or establish concrete conclusions at this point. For this reason, the goal of this review will be to provide a general update on the current status of metal NP toxicological assessment with an emphasis on commonly used metal NPs in medical, consumer, industrial and military applications. The compositions of the metal-based NPs covered in this review include the following: aluminum (Al), aluminum oxide (Al_2O_3), gold (Au), silver (Ag), copper (Cu), iron (Fe), iron oxide (Fe_2O_3, Fe_3O_4), manganese (Mn), manganese oxide (MnO), silicon dioxide (SiO_2), titanium dioxide (TiO_2), zinc (Zn), zinc oxide (ZnO) as well as other metal-oxides such as ceria (CeO_2), nickel oxide (NiO), and zirconia (ZrO_2).

Although not explored in this review, carbon nanotubes are receiving considerable attention for myriad applications.[76–78] However, it is worth mentioning here that the reactive metallic (i.e., Fe, Co, and Ni) residues remaining from the catalyst particles used in the synthesis of carbon nanotubes (CNTs) have been implicated in their toxicity and generation of reactive oxygen species (ROS).[79–81] Furthermore, luminescent semiconductor nanocrystals, referred to as quantum dots (QDs), have gained interest over the past several years for coupling with biomolecules for the imaging of biological systems.[82,83] However, great concern has arisen for leaching of the heavy metals (Cd, Zn, Se, etc.), which compose the core of the QDs, the generation of ROS,[84] or pro-inflammatory cytokines.[85,86] Therefore, all NPs are undergoing heavy scrutiny to determine if their benefits outweigh their risks, and their applications are briefly summarized in Table 1.

Safety Issues of Metal-based Nanoparticles

Although great strides have been made in the worldwide production and use of metal-based NPs, there is a serious lack of information about the impact of NPs on human health and environment, especially the potential for NP-induced toxicity.[87] Preliminary reports of the inherent toxicity of some NPs are available and indicate that they can affect biological behavior at the organ, tissue, cellular, subcellular, and protein levels. However, more fully understanding the basis of NP toxicity is a requisite to the completion of occupational and environmental exposure risk-assessments, which must be overcome before large-scale production of NPs can be safely and efficiently applied in the field of medicine. Similar to fumes, ionic, or bulk forms of metals, it is

TABLE 1 | Selected Applications of Metal Nanoparticles

Nanoparticle	Abbreviation	Application
Aluminum	Al	Fuel additive/propellant, explosive, wear resistant coating additive
Gold	Au	Cellular imaging, photodynamic therapy
Iron (oxide)	Fe, Fe_3O_4, Fe_2O_3	Magnetic imaging, environmental remediation
Silica	SiO_2	Fabrication of electric and thermal insulators, catalyst supports, drug carriers, gene delivery, adsorbents, molecular sieves, and filler materials
Silver	Ag	Antimicrobial, photography, batteries, electrical
Copper	Cu	Antimicrobial (i.e., antiviral, antibacterial, antifouling, antifungal), antibiotic treatment alternatives, nanocomposite coating, catalyst, lubricants, inks, filler materials for enhanced conductivity and wear resistance
Cerium (oxide)	CeO_2	Polishing and computer chip manufacturing, fuel additive to decrease emissions
Manganese (oxide)	Mn	Catalyst, batteries
Nickel (oxide)	Ni	Conduction, magnetic properties, catalyst, battery manufacturing, printing inks
Titanium dioxide	TiO_2	Photocatalyst, antibacterial coating, sterilization, paint, cosmetics, sunscreens
Zinc (oxide)	Zn, ZnO	Skin protectant, sunscreen

foreseeable that metal-based NPs could contribute to adverse health conditions including concentration-dependent alterations in gene expression and other critical physiological processes due to improper intracellular trafficking and accumulation at toxic concentrations.[88–90] In the following sections, the current testing methods and potential routes of NP exposure and biodistribution will be considered prior to data on current *in vitro* and *in vivo* toxicity assessments.

TOXICITY OF NANOPARTICLES

The toxicity of NPs is being addressed by a number of standardized approaches with *in vitro*, *in vivo* as well as detailed genomic or biodistribution studies. *In vitro* models (i.e., cells in culture) can act as a pre-screening tool for NP bio-effects. *In vitro* studies in cultured cells have several advantages, including rapid results with low cost and decreasing the need for animal use, although they (*in vitro* tests) are not performed as a replacement for *in vivo* models. Moreover, the experiments can be repeated several times to get confirmed and statistically significant results. However, it has been shown that NPs may produce *in vitro* toxicity in some cell-based assays, but not in others. This may be a result of interference with the chemical probes, differences in the innate response of particular cell types, or other factors. Therefore, it is suggested that the biological activities of NPs should be assessed by multiple cell-based assays with several cell types and multiple doses,[91] to confirm the results between laboratories[92] and to rely on animal models to more realistically study the suitability of NPs for applications. In addition to *in vitro* and *in vivo* studies, microarray and real-time reverse transcription (RT) polymerase chain reaction (PCR) are very sensitive and reliable methods for gene expression analyses, which can measure the changes in the expression levels of thousands of genes simultaneously under a wide variety of experimental conditions.

The various interactions of NPs with fluids, cells, and tissues need to be considered from the route of entry through the wide range of possible pathways ending at potential target organs. NPs may be able to enter the body via routes such as the gastrointestinal tract,[93] lungs,[94,95] injection into the blood stream, and passage through the skin.[6,7] Although the epidermis is an excellent barrier to protect the body from external insult, NPs such as TiO_2 or ZnO in sunscreens have been shown to penetrate and be retained within the human stratum corneum and into some hair follicles. The fear is that if these NPs reach the capillary junction, either through penetration of the epidermis or through compromised skin,[6,7,86] than they may pose a systemic health threat.[32,96] After inhalation of NPs, cells in the respiratory system such as macrophages and epithelial cells that line the lungs may come into direct contact with NPs. Further translocation to the lymphatic system could induce secretory immune responses. In contrast, when NPs enter the circulation, they may influence endothelial cell membrane toxicity and/or disrupt the tight junctions of the blood–brain barrier and gain access into the cerebral environment.[97]

Following systemic administration, NPs may be able to penetrate very small capillaries throughout the body and efficiently distribute to certain tissues.[98] In this case, NPs passing through epithelia and biological membranes can potentially affect the physiology of any cell in the body.[84,99] After passing through the body, it is anticipated that NPs would be filtered through excretory organs in the body such as the liver and kidney. Ag and Cu NPs have demonstrated a greater potential to travel through the organ systems compared to larger materials[100,101] and may not be detected by normal phagocytic defenses, allowing them to gain access to the blood or cross the blood–brain barrier into the nervous system. Furthermore, Ag, Cu, and Al NPs may induce oxidative stress and generate free radicals that could disrupt the endothelial cell membrane.[97] This disturbance may cause blood–brain barrier dysfunction resulting in the entry of NPs into the central nervous system.

In addition to passing through the blood–brain barrier, NPs may have reproductive consequences after penetrating the blood–testis barrier[84] and specifically the Leydig cells.[102–106] Li et al.[103] recently demonstrated that *in utero* exposure to NPs contained in diesel exhaust affects testicular function by suppressing the production of testosterone.[104] Furthermore, it has recently been demonstrated that certain metal NPs reduce spermatogonial stem cell proliferation *in vitro*.[107] However, the potential for NPs to interact with cells of the reproductive system and disrupt normal function is still not well studied or understood. Therefore, it is anticipated that NPs can have far-reaching implications on human health and will continue to be evaluated through a variety of scientific methods. In the following sections, varieties of NP-induced bio-effects are organized according to applications and elemental compositions with an emphasis on the methods used to assess toxicity (i.e., *in vitro*, *in vivo*, genomic, and biodistribution studies). In studies where multiple NP compositions were simultaneously tested, the results were grouped together for clarity.

Aluminum Nanoparticles

Due to the great potential use of Al NPs in military applications such as coatings, propellants, and fuels, the likelihood of exposure to soldiers and other military personnel is increasing. Wagner et al.[108] examined the cellular interaction of aluminum oxide and aluminum nanomaterials, including their effect on cell viability and cell phagocytosis, with reference to particle size and chemical composition. Experiments were performed to characterize initial *in vitro* cellular effects of rat alveolar macrophages (NR8383) after exposure to aluminum oxide nanoparticles (Al_2O_3NP at 30 and 40 nm) and aluminum metal NPs containing a 2–3 nm oxide coat (Al NP at 50, 80, and 120 nm). Characterization of the nanomaterials, both as received and *in situ*, was performed using transmission electron microscopy (TEM), dynamic light scattering (DLS), laser Doppler velocimetry, and/or CytoViva150 Ultra Resolution Imaging (URI). Particles showed significant agglomeration in cell exposure media using DLS and the URI as compared to primary particle size in TEM. Cell viability assay results indicate a marginal effect on macrophage viability after exposure to Al_2O_3 NP at doses of 100 μg/mL for 24 h of continuous exposure. In contrast, Al NP produced significantly reduced viability after 24 h of continuous exposure with doses from 100 to 250 μg/mL. Cell phagocytotic ability was significantly hindered by exposure to 50, 80, or 120 nm Al NPs at 25 μg/mL for 24 h, but the same concentration (25 μg/mL) had no significant effect on the cellular viability. However, no significant effect on phagocytosis was observed with Al_2O_3 NP. In summary, these results show that Al NP exhibit greater toxicity and more significantly diminish the phagocytotic ability of macrophages after 24 h of exposure when compared to Al_2O_3 NP (Figure 1). Furthermore, toxicity to Al NPs in mammalian germline stem cells indicated by significant increases in lactate dehydrogenase (LDH) leakage and the induction of apoptosis at concentrations from 1 to 100 μg/mL after 48 h has been shown.[107]

Gold Nanoparticles

Although there are an increasing number of studies to examine the potential toxicity of Au NPs prior to widespread clinical application, the reported data thus far are highly dependent upon the synthesis methods and resulting Au NP size, shape, surface chemistry, and surface charge. Pan et al.[109] recently investigated the size-dependent toxicity of Au NPs (0.8–15 nm) in four different cells lines demonstrating that one of the smallest NPs tested (1.4 nm) had the greatest toxicity

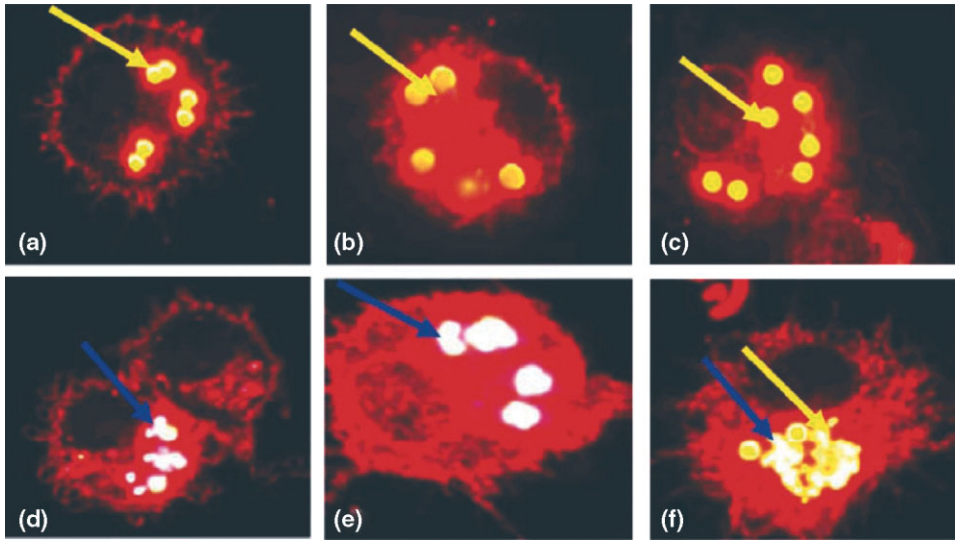

FIGURE 1 | Microscopic observation of Al_2O_3 nanoparticles (NPs) and Al NPs phagocytized by alveolar macrophages (AM). Various representative images (a–f) were taken during phagocytosis with the Olympus IX71 inverted fluorescent microscope attached with an advanced high illuminating system. Cells were exposed to Al_2O_3NPs and Al NPs at 5 or 25 μg/mL for 24 h. Fluorescent latex beads (2 μm) were given to the cells after exposure. The beads appear as bright globular areas in the cells and were dosed at a 10:1 ratio (10 beads for every cell) for 6 h. Macrophages and beads phagocytized by macrophages were counted to obtain a phagocytosis index (PI). PI defined as % macrophages that take in beads × average number of beads taken in by a positive macrophage. (a) No exposure to Al NPs (control); (b) AM exposed to 25 μg/mL of Al_2O_3 NPs 30 nm; (c) AM exposed to 25 μg/mL of Al_2O_3NPs 40 nm; (d) AM exposed to 5 μg/mL of Al NPs 50 nm; (e) AM exposed to 5 μg/mL of Al NPs 80 nm; (f) AM exposed to 5 μg/mL of Al NP 120 nm. Yellow arrows indicate the uptake of fluorescent latex beads. Blue arrows indicate the Al particles uptake (Reprinted with permission from Ref 108. Copyright 2007 IOP Publishing).

compared to other Au NPs with sizes up to 15 nm.[109] Furthermore, the larger-sized particles exhibited no toxicity even at concentrations as high as 6.3 mM. An *in vivo* toxicity study of spherical colloidal Au NPs intravenously injected into mice showed that the smaller particles (10–50 nm) caused more toxicity compared to the larger particles (100–200 nm), although the surface chemistry was not specifically mentioned.[103] Yen et al.[110] reported that spherical Au NPs produced by the grinding and vaporizing of bulk gold (2.8, 5.5, and 38 nm in size) were toxic and induced immunological responses with the smaller Au NPs up-regulating the expression of pro-inflammatory genes interleukin-1 (IL-1), interleukin-6 (IL-6), and tumor necrosis factor (TNF-α).[110]

Wang et al.[111] studied the effect of shape on toxicity and found that CTAB-coated Au nanorods were more toxic than spherical Au NPs (~30 nm) to human HaCaT keratinocytes. The effects of Au NP surface charge on toxicity were examined by studying cationic (amine) and anionic (carboxyl) spherical Au NPs, ~2 nm in size on Cos-1 cells, red blood cells, and *E. coli* bacteria.[112] The results indicated that the cationic or positively charged Au NPs exhibited more toxic effects compared to the anionic or negatively charged Au NPs of the same size.[112] Li et al.[113] recently showed that while citrate reduced, average 20-nm-sized Au NPs were not toxic to lung fibroblasts, they did produce significant amounts of oxidative DNA damage and down-regulated the expression of DNA damage and cell-cycle genes. Furthermore, Pernodet et al.[114] illustrated that 14-nm Au NPs cause abnormal actin and extracellular matrix in dermal fibroblasts. These abnormal proteins, in turn, cause a major decrease in cell proliferation, adhesion, and motility.

In vivo studies have reported that Au NPs can cross the small intestine by persorption and further distribute into the blood, brain, lung, heart, kidney, spleen, liver, intestine, and stomach.[115] Single Au NPs (11.6 ± 0.9 nm in diameter) passively diffused into the chorionic space of embryos via their chorionic pore canals and continued through chorionic space into the inner mass of embryos. Embryos chronically incubated with 0.025–1.2 nM Au NPs for 120 h resulted in 74% developing into normal zebrafish, ~24% dying, and ~2% displaying deformities.[116]

Despite the seemingly bleak results for Au NP toxicity, there are many other studies that report the nontoxic and nonreactive nature of Au NPs toward cells of the body. For example, Connor et al.[117] showed that spherical Au NPs (4, 12, and 18 nm) with a variety of surface modifiers were not toxic to human leukemia cells. Shukla et al.[118] reported that spherical 3.5 nm Au NPs capped with lysine were not toxic to macrophages at concentrations up to 100 μM after 72 h of exposure and did not elicit the secretion of pro-inflammatory cytokines TNF-α or IL-1β. These two studies suggest that synthesis conditions and resultant surface chemistry of Au NPs may play a major role in modifying the biological response. A recent review on Au NPs can be consulted for further references.[119]

Silicon and Silica Nanoparticles

Vascular endothelial cells that have internalized silicon microparticles maintain cellular integrity as demonstrated by cellular morphology, viability, and intact mitotic trafficking of vesicles bearing silicon microparticles. The presence of gold or iron oxide NPs within the porous matrix did not alter the cellular uptake of particles or the viability of endothelial cells subsequent to engulfment of microparticles. The finding that mitotic sorting of endosomes is unencumbered by the presence of nanoporous silicon microparticles advocates the use of silicon microparticles for biomedical applications.[120]

Yu et al.[121] examined the uptake, localization, and cytotoxic effects of well-dispersed amorphous SiO_2 NPs in mouse keratinocytes (HEL-30). Mouse keratinocytes were exposed for 24 h to various concentrations of amorphous SiO_2 NPs in homogeneous suspensions of average size distribution (30, 48, 118, and 535 nm SiO_2) and then assessed for uptake and biochemical changes. Results of TEM revealed that all sizes of silica were taken up into the cells and localized into the cytoplasm. The LDH assay shows that LDH leakage was dose- and size-dependent with exposure to 30 and 48 nm NPs. However, no LDH leakage was observed for either 118 or 535 nm particles. The mitochondrial viability assay (MTT) showed significant toxicity for 30 and 48 nm at high concentrations (100 μg/mL) compared to the 118 and 535 nm particles. Further studies were carried out to investigate if cellular-reduced GSH and mitochondria membrane potential are involved in the mechanism of SiO_2 toxicity. The redox potential of cells (GSH) decreased significantly at concentrations of 50, 100, and 200 μg/mL with 30 nm NP exposures. However, SiO_2 NPs larger than 30 nm showed no changes in GSH levels. ROS formation did not show any significant change between controls and the exposed cells. In summary, amorphous SiO_2 NPs below 100 nm induced toxicity, suggesting that the size of the particles is critical to produce biological effects.

Brown et al.[122] dosed normal human mesothelial cells with 100 nm SiO_2 spheres at a concentration of 26.7 μg/mL and reported LDH leakage as 3% after 24 h exposure. Thibodeau et al.[123] studied

SiO_2-induced apoptosis in the mouse alveolar macrophages to investigate lung disease characterized by pulmonary fibrosis. The authors reported that mitochondrial depolarization and Caspase 3 and 9 activation contributed to apoptosis when the cells were exposed to silica. The role of ROS was investigated in their study, but it was not apparent.

Kim et al.[84] treated mice with silica-coated magnetic nanoparticles (MNPs) for 4 weeks and found NPs in almost all organs in a time-dependent manner. Most of the NPs were taken up by the liver and then redistributed to other organs (e.g., spleen, lungs, heart, and kidney). They also reported that NPs (<50 nm) reached the brain and testes after bypassing the blood–brain barrier and blood–testis barriers, respectively, without inducing any apparent toxicity. These results suggest that MNPs exhibit potential biological characteristics to act as vectors for gene transfer and gene/drug delivery.

Silver Nanoparticles

As previously mentioned, Ag NPs have found widespread use in consumer and medical applications as antimicrobials. Furthermore, the unique plasmon-resonant optical scattering properties of Ag NPs are finding use in applications for signal enhancement, optical sensing, biomarkers, and *in vivo* imaging agents.[29] However, the use of Ag NPs in the imaging of neural tissue and cells, in particular, raises concerns over the possibility of contributing to neurodegenerative diseases (e.g., Parkinson's and Alzheimer's) due to their ability to produce ROS and oxidative stress.[124,125] Indeed, studies in our laboratory have shown dose- and size-dependent toxicity, largely mediated through oxidative stress, induced by Ag NPs in neuroendocrine cells, liver cells, lung cells, and germline stem cells at concentrations between 5 and 100 μg/mL after 24 h of exposure.[107,126–129] Carlson et al.[129] evaluated size-dependent cellular interactions of known biologically active Ag NPs (15, 30, and 55 nm) in alveolar macrophages. Alveolar macrophages provide the first line of defense against foreign debris in the lung and were studied for their potential role in initiating oxidative stress. *In vitro* exposure produced morphologically abnormal sizes and adherence characteristics with significant NP uptake at high doses after 24 h. Toxicity evaluations using mitochondrial and cell membrane viability along with ROS showed a dose-dependent decrease in cell viability. A more than 10-fold increase of ROS levels in cells exposed to 50 μg/mL 15-nm Ag NPs suggests that the toxicity is likely to be mediated through oxidative stress. In addition, activation of the release of traditional inflammatory mediators was examined by measuring the levels of cytokines/chemokines, including TNF-α, macrophage inhibitory protein (MIP-2), and IL-6, released into the culture media. After 24 h of exposure to 15 nm Ag NPs, a significant inflammatory response was observed by the release of TNF-R (TNF-alpha), MIP-2, and IL-1β. However, there was no detectable change in the level of IL-6 upon exposure to Ag NPs.

The possibility of using Ag NPs as biolabels was explored with Neuro-2A cells.[128] Schrand et al. found that two Ag NPs, with different surface chemistries (hydrocarbon vs polysaccharide), produced strong optical labeling with high illumination light microscopy after 24 h of incubation. This was due to the excitation of plasmon resonance by both types of Ag NPs. Both types of Ag NPs were also bound to the exterior surface of the Neuro-2A cells and were internalized into intracellular vacuoles. However, ROS production, degradation of mitochondrial membrane integrity, disruption of the actin cytoskeleton, and reduction in proliferation after stimulation with nerve growth factor were found after incubation with Ag NPs at concentrations of 25 μg/mL or greater, with a more pronounced effect produced by the hydrocarbon-based Ag NPs in most cases. Representative electron microscope images of ~25 nm Ag NPs are shown in Figure 2.[128]

In vivo studies with adult Sprague–Dawley rats dosed with Ag NPs (60 nm) at low (30 mg/kg), medium (300 mg/kg), and high doses (1000 mg/kg) for 28 days showed significant dose-dependent changes in plasma alkaline phosphatase (ALP) and blood cholesterol indicating that these NPs could damage the liver.[130] However, Ag NPs did not induce genetic toxicity in either male or female rat bone marrow.[130] Furthermore, a dose-dependent accumulation of Ag NPs was observed in all tissues examined. In particular, a gender-related difference in the accumulation of silver was recorded in the kidneys, with a twofold increase in the female kidneys when compared with the male.[130] Male and female rats exposed to respiratory contact with $1.73 \times 10^4/cm^3$ (low), $1.27 \times 10^5/cm^3$ (medium), and 1.32×10^6 particles/cm^3 (high) doses of Ag NPs for 6 h/day, 5 days/weeks for 4 weeks did not yield significant changes in body weight, hematology, or blood biochemical parameters. However, histopathological examination of the liver revealed cytoplasmic vacuolization and hepatic focal necrosis in some of the Ag NP-treated rats.[131] In other studies, Ag NPs were implanted into the back muscles of rats for 180 days resulting in serious inflammation and granuloma formation. The small size of the NPs and

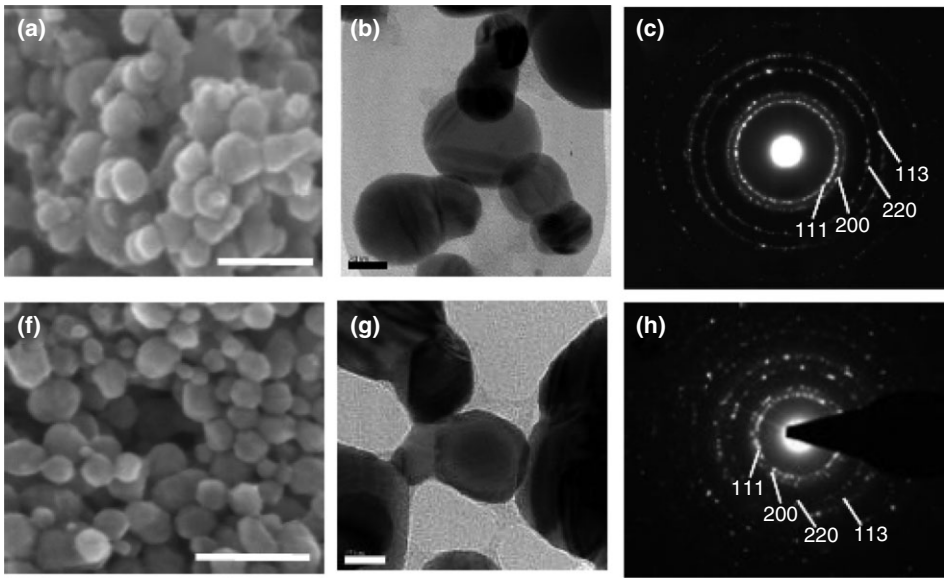

FIGURE 2 | Electron microscopy characterization of hydrocarbon-processed 25 nm silver nanoparticles (Ag25) and polysaccharide-coated silver nanoparticles (Ag25Disp). (a–c) Ag25 and (f–h) Ag25Disp. (a, f) Scanning electron microscope images with scale bars 100 nm; (b, g) transmission electron microscope images with scale bars 20 nm; (c, d) selected area diffraction patterns (Reprinted with permission from Ref 128. Copyright 2008 IOP Publishing Limited).

large surface area to volume ratio resulted in a large number of macrophages around the implanted particles. Numerous Ag NPs in the macrophage cell cytoplasm were also reported.[100] In a separate study, a concentration-dependent increase in mortality and hatching delay was recorded in Ag NP-treated embryos of zebrafish.[132] Furthermore, abnormal body axes, twisted notochord, slow blood flow, pericardial edema, and cardiac arrhythmia were also found in zebrafish after Ag NP exposure. TEM of the embryos demonstrated that NPs were distributed in the brain, heart, yolk, and blood of embryos as evident from the electron dispersive X-ray analysis (EDS) (Figure 3). These results indicated that Ag NPs induced a dose-dependent toxicity in zebrafish embryos.[132] In our recent studies, we have found that Ag NPs (25 nm) increased ROS production both *in vitro* and *in vivo* and simultaneously altered gene expression in the frontal cortex of mice.[133] In particular, glutathione peroxidase genes were down-regulated, causing apoptosis and neurodegeneration.[133] In a similar study, we found that 100–1000 mg/kg doses of 25 nm Ag NPs caused significant alterations in oxidative stress and antioxidant defense arrays in the caudate, frontal cortex, and hippocampus of mice. These results suggest that neurotoxicity occurs by altering gene expression and generating free radical-induced oxidative stress, producing apoptosis and neurotoxicity.[133] Furthermore, it is reported that Ag NPs exerted considerable toxicity by decreasing reproduction potential in *Cenorhabditis elegans* with increased gene expression of sod3 and daf12, which might be related to Ag NP-induced reproduction failure in *C. elegans*.[134]

Copper Nanoparticles

Copper nanoparticles (Cu NPs) have been heavily researched as candidates for novel antimicrobial (i.e. antiviral, antibacterial, antifouling, and antifungal) applications as biocides, antibiotic treatment alternatives, and nanocomposite coatings.[40,41] However, Cu NPs have demonstrated severe toxicological effects including heavy injuries in the kidney, liver, and spleen of mice after ingestion, which are readily evident via histological analysis[135,136] (Figure 4). The oral LD_{50} of Cu NP (23.5 nm) in mice was reported to be 413 mg/kg, which is considered moderate toxicity similar to Zn powder.[135,137] In contrast, Cu microparticles (17 μm) did not produce similar effects and were classified as nontoxic with LD_{50} values of >5000 mg/kg. Moreover, glomerulitis, degeneration, and necrobiosis of renal tubules were observed in the mice exposed to Cu NPs, but not in the mice exposed to Cu microparticles indicating that particle size and surface area are important material characteristics from a toxicological perspective. Supporting evidence for the more efficient deposition of Cu NPs compared to micro-sized particles in renal tissues was demonstrated in an associated study by Meng et al.[41] They proposed that once inside the kidney, Cu NPs reacted with gastric juices and were converted to more toxic cupric ions.[136]

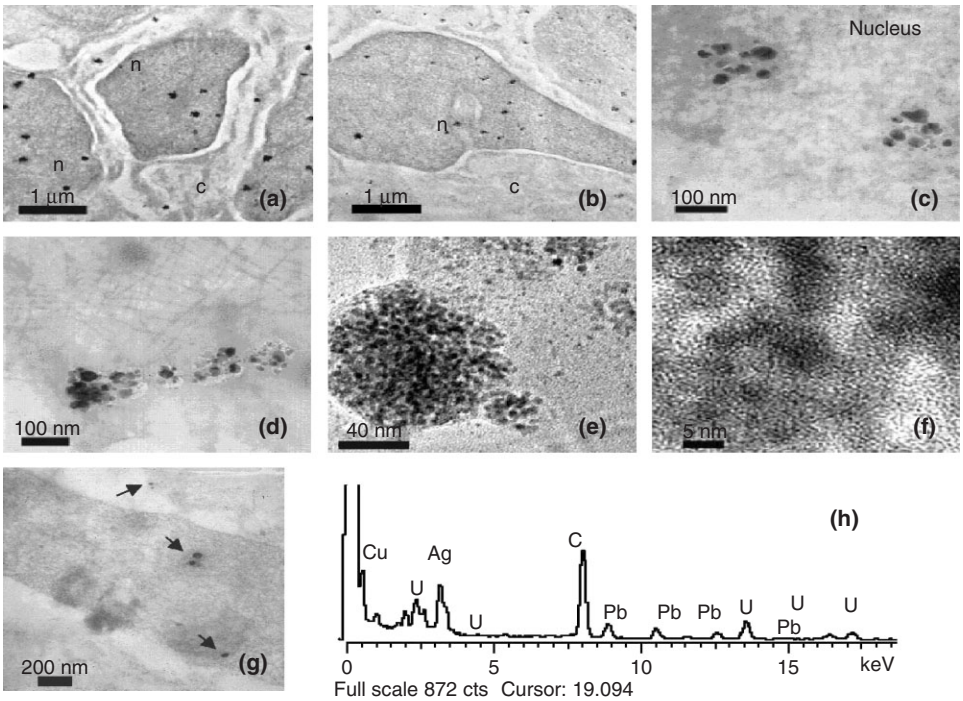

FIGURE 3 | Transmission electron microscopy images of ultrathin sections of the zebrafish embryos treated with 25 μg/mL of Ag-BSA nanoparticles (NPs). (a) Deposition of the Ag NPs in the cytoplasm and (b) nucleus of the cells near the trunk and tail, respectively. Images were captured using a JEOL JSM 3010F. The nucleus is indicated by 'n' and cytoplasm by 'c'. (c) Magnified images of the nucleus show NP deposition. (d) Clumps of NPs were seen near the epithelium. (e) Low magnification images of the heart, showing dark spots containing NPs. (f) Magnified images from heart confirming the presence of NPs. The lattice plane identifies NPs. (g) Sections of brain showing the presence of NPs. (h) EDS of embryos showing the presence of Ag (Reprinted with permission from Ref 132. Copyright 2008 IOP Publishing Limited).

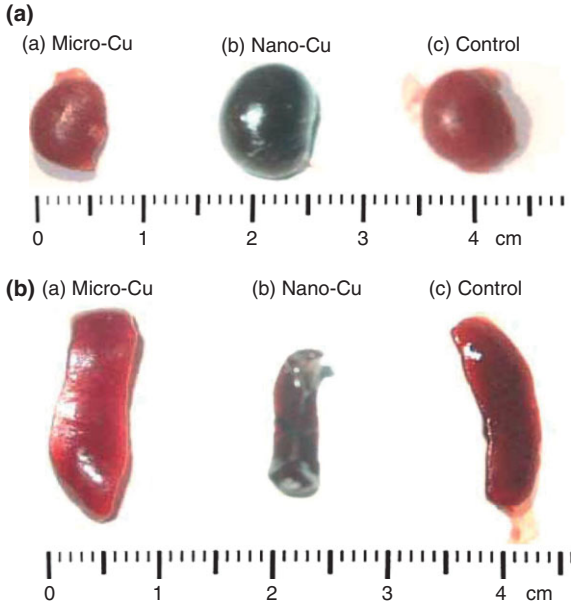

FIGURE 4 | (A) The appearance of mouse kidneys in various treatment groups: (a) micro-Cu (1077 mg/kg), (b) nano-Cu (1080 mg/kg) and (c) the control. (B) The appearance of mouse spleens in various treatment groups: (a) micro-Cu (1077 mg/kg), (b) nano-Cu (1080 mg/kg) and (c) the control (Reprinted with permission from Ref 135. Copyright 2006 IOP Publishing).

The acute toxicity of Cu NPs (80 nm) (1.5 mg/L; LC_{50}) was reported in zebrafish, showing a decrease in gill Na^+/K^+-ATPase activity.[138] Cu NP treatment also decreased blood urea nitrogen (BUN) levels and increased plasma alanine amino transferase (ALAT) levels. Additionally, dose-dependent damage of gill lamellae characterized by proliferation of epithelial cells, as well as edema of primary and secondary gill filaments, was observed after Cu NP treatment (Figure 5), indicating that the gill was the primary target organ for Cu NP in zebrafish.[138] In zebrafish exposed to Cu NPs, RT–PCR results showed higher levels of gene expression changes compared to $CuSO_4$-exposed fish. Furthermore, cluster analysis of these gene microarrays demonstrated that the transcriptional response induced by Cu NP was highly divergent.[138]

Titanium Dioxide Nanoparticles

TiO_2 is one of the most widely manufactured nanomaterials, synthesized into three common nanoarchitectures: anatase (7–10 nm), rutile (15–20 nm), and nanotubes (10–15 nm diameters, 70–150 nm length) in addition to rods and other shapes.

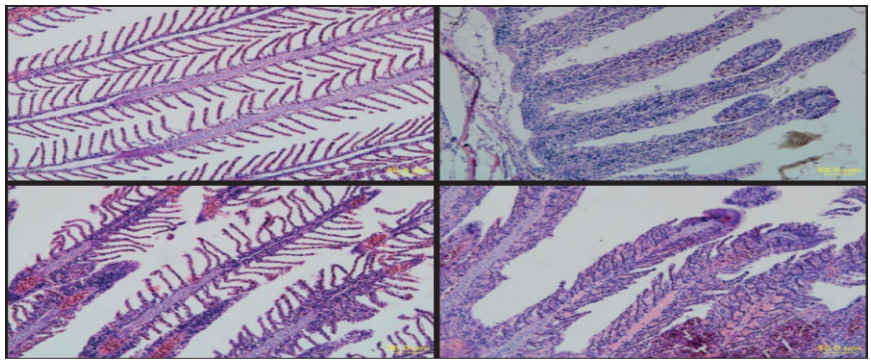

FIGURE 5 | Micrographs showing gill injury induced by 48 h copper exposure. Soluble copper and nanocopper induced [sic] dramatic changes in gill morphology. Clockwise from top left: Control, 0.25 mg/L soluble Cu^{2+}, 1.5 mg/L nanocopper, 0.25 mg/L nanocopper (Reprinted with permission from Ref 138. Copyright 2007 American Chemical Society).

Applications of TiO_2 NPs range from paints to sunscreen and cosmetic additives to surface coatings. In general, toxicity studies of TiO_2 have shown the induction of inflammatory responses and ROS in a variety of cell types and tissues.[139–145] However, it has been difficult to determine the physicochemical properties of the TiO_2, which were responsible for the effects since many early studies did not take into account the different sizes or crystal structures (anatase and rutile). For example, smaller 29-nm TiO_2 NPs of unknown crystal structure increased inflammation and altered macrophage chemotactic responses in rat lungs, when compared to larger 250-nm TiO_2 NPs.[139] Similarly, smaller TiO_2 NPs of varying compositions produced oxidative damage in a human bronchial epithelial cell line.[143] To answer the question of whether the size of TiO_2NPs (composed of the same crystal structure) or if the crystal structure of TiO_2 NPs (with similar primary diameters) was a determining factor in TiO_2-induced toxicity,[145] Braydich-Stolle et al.[145] examined the controlled forms of TiO_2 NPs in the mouse keratinocyte cell line HEL-30. They found that 100% anatase TiO_2 NPs, regardless of size, induced cell necrosis, whereas the rutile TiO_2 NPs initiated apoptosis by the formation of ROS.[145] Their results were in agreement with the earlier studies by Sayes et al.[142] demonstrating that anatase TiO_2 was more toxic than rutile TiO_2. Other studies have evaluated the toxicity of TiO_2 NPs using the human bronchial epithelial cell line (BEAS-2B) at different concentrations (5, 10, 20, and 40 μg/mL).[146] Cell death, ROS increase, reduced glutathione (GSH) decrease, and the induction of oxidative stress-related genes (such as heme oxygenase-1, thioredoxin reductase, glutathione-S-transferase, catalase, and hypoxia-inducible gene) were observed. Furthermore, the elevation of inflammation-related genes such as IL-1, IL-6, IL-8, TNF-α, C-X-C motif ligand 2, and IL-8 gene were induced through a p38 mitogen-activated protein kinase pathway and/or extracellular signal pathway.[146]

In contrast, an *in vivo* study in rats found that nano-sized TiO_2 rods/dots produced inflammatory responses that were not different from the pulmonary effects of larger TiO_2 particles.[140] Similarly, Renwick et al.[147] looked at carbon black and TiO_2 particles in the fine and ultrafine size ranges, and they found that neither compound was directly toxic to macrophages, but did significantly reduce the ability of the cells to phagocytose other particles. This decrease in phagocytosis was more prevalent in the ultrafine particles as compared to their macro-sized counterparts.

Due to the low toxicity of TiO_2, Wang et al.[148] treated mice with a large dose of 5 g/kg body weight. The compound was administered by a single dose through oral route according to the OECD guideline no. 420. The authors reported changes in serum biochemical parameters (aspartate amino transferase), ALAT, LDH, and pathology (hydropic degeneration around the central vein and spotty necrosis of hepatocytes) of the liver, indicating hepatic injury in female mice treated with TiO_2 NPs (25 and 80 nm) compared to fine (155 nm) TiO_2 NPs. Nephrotoxicity (increased BUN level), pathology changes in kidneys, and accumulation of TiO_2 were observed in the liver, spleen, kidneys, and lung tissues, indicating that the NP could be transported to other tissues and organs after uptake in the gastrointestinal tract (Figure 6).

In an alternative animal model, the fully human autologous modular immune *in vitro* construct (MIMIC) immunological construct was utilized to predict TiO_2 NP immunogenicity. Cumulatively, treatment with TiO_2 NPs in the MIMIC system led to elevated levels of pro-inflammatory cytokines and increased maturation and expression of co-stimulatory molecules on dendritic cells.

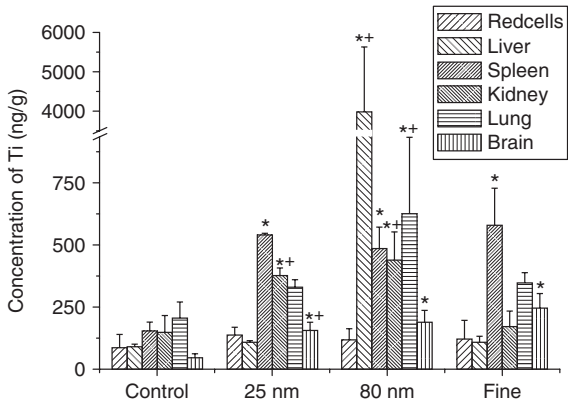

FIGURE 6 | Titanium concentration in different tissue types of female mice 2 weeks post-exposure. Varying sizes of titanium dioxide (TiO_2) nanoparticles (NPs) were administered. ∗ Represents significant difference from the control group (Dennett's, $p < 0.05$), and + represents significant difference from the fine 155 nm TiO_2 group (Student's, $p < 0.05$) (Reprinted with permission from Ref 148. Copyright 2007 Elsevier).

Additionally, these treatments effectively primed activation and proliferation of naive CD4-T cells in comparison to dendritic cells treated with micrometer-sized (>1 μm) TiO_2, characteristic of an *in vivo* inflammatory response.[149]

Cerium Oxide Nanoparticles

Nano-sized cerium oxide (CeO_2) has found increasing use in polishing and computer chip manufacturing[150,151] as well as an additive to decrease diesel emissions.[152] Different sizes of CeO_2 (15, 25, 30, 45 nm) NPs caused toxicity, ROS increase, GSH decrease, and induced oxidative stress-related genes (such as heme oxygenase-1, catalase, glutathione-S-transferase, and thiorexoxin reductase) in cultured human lung epithelial cells (BEAS-2B). It was reported that the increased ROS by these NPs triggered the activation of cytosolic Caspase 3 and chromatin condensation, causing toxicity via the apoptotic process.[146] The morphological changes to these cells such as chromosome condensation are shown in Figure 7. In contrast, other studies with ceria nanostructures showed high biocompatibility,[153] conferred radioprotection to normal cells compared to no protection for tumor cells,[154] and prevented retinal degeneration induced by intracellular peroxidases.[101] This apparent discrepancy may be due to the surface oxidation state of nanoceria to scavenge superoxides or act in a catalytic manner. Alternatively, Rothen-Rutishauser et al.[153] exposed A549 lung cells directly to flame-spray synthesized CeO NPs for 10–30 min and did not notice any significant change in LDH leakage or cell morphology, but did find decreases in the mean total lamellar body volume per cell, reduction of cell–cell contacts and a significant increase in 8-oxoguanine positive cells indicative of the secretion

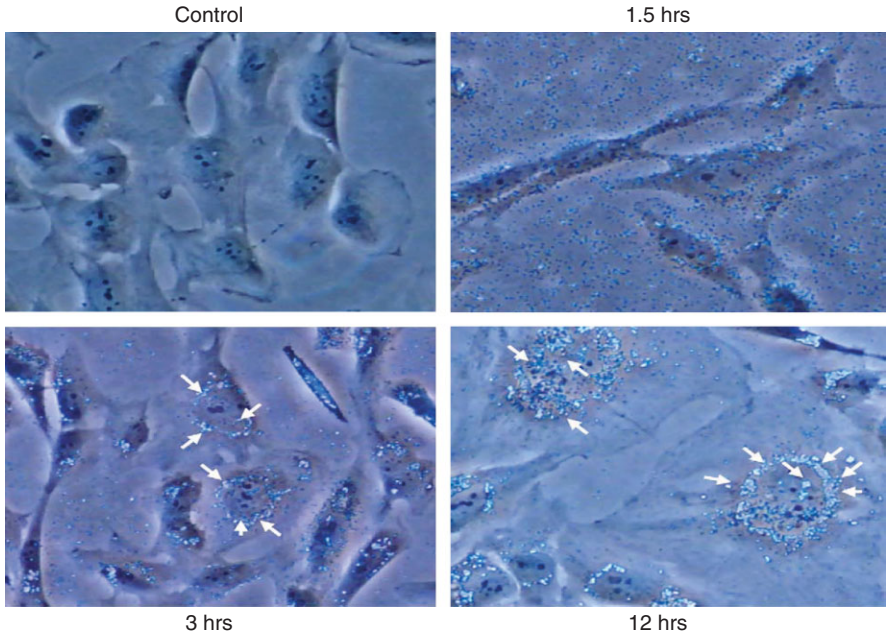

FIGURE 7 | Microscopic observation of the cells treated with cerium oxide nanoparticles. Aggregates of cerium oxide nanoparticles with bright microscopic images were localized in the perinuclear region of nucleus. The images of aggregates were enlarged with the increase in exposure time to form a ring like shape. Arrows show the aggregates of cerium oxide nanoparticles in the cells (Reprinted with permission from Ref 146. Copyright 2008 Elsevier).

of surfactant as a protective response and NP-induced oxidative stress resulting in altered gene expression.[155]

Comparative *In Vitro* Toxicity Studies

Due to the ease of duplication and rapid results produced by *in vitro* toxicity tests, many studies have simultaneously examined multiple compositions of metal NPs (i.e., Al, Al_2O_3, Ag, Cu, Fe_2O_3, Fe_3O_4, Mn, MnO_2, MoO_3, Si_3N_4, TiO_2, CrO_3, ZnO, and ZrO_2) under similar experimental conditions. Through these studies, a better understanding of the comparative toxicity of NPs, based on elemental composition and other physicochemical properties, can be gleaned. In general, Zn-based NPs have demonstrated greater *in vitro* toxicity compared to many other metal NP compositions such as Ag and Cu[92,156] (Table 2). For example, Jeng et al.[155] examined the relative toxicity of TiO_2, ZnO, Fe_3O_4, Al_2O_3, and CrO_3 NPs with primary sizes ranging from 30 to 45 nm and concentrations up to 200 μg/mL in Neuro-2A cells.[157] They found that ZnO was highly toxic, whereas Al_2O_3 was moderately toxic and Fe_3O_4 and TiO_2 exhibited slight toxicity at high concentrations compared to low toxicity for CrO_3 NPs. In an animal study, Wang et al.[156] evaluated the acute oral toxicity of a very high dose of nano Zn powder (58 nm; 5 g/kg) in mice and found severe symptoms of lethargy, vomiting, and diarrhea along with significant elevation in biochemical parameters like ALAT, ALP, and LDH. Other studies have also shown the high potential for toxicity after exposure to Zn NPs.[158–162] Interestingly, doping ZnO with Fe could reduce toxicity by changing the material matrix to slow Zn^{2+} release.[158]

In studies where Zn-based NPs were not included in the compositions tested, Ag NPs were found to be highly cytotoxic. For example, the cellular toxicity of Ag (15 and 100 nm), MoO_3(30 and 150 nm), Al (30 and 103 nm), Fe_3O_4 (30 and 47 nm), and TiO_2 (40 nm) was assessed in comparison to larger particles in the rat liver cell line (BRL-3A).[127] These results indicated that Ag was highly toxic, MoO_3 moderately toxic, and Fe_3O_4, TiO_2, Al, and MnO_2 displayed lower relative toxicities. In comparison, 40 nm MnO NPs and ionic manganese (Mn^{2+}) were less toxic than 15 nm Ag NPs in PC-12 cells.[126] In a similar study in PC-12 cells, Wang et al.[166] examined 40 nm Mn NPs, 15 nm Ag NPs, and 90 nm Cu NPs at 10 μg/mL doses. They found that Mn and Cu NPs depleted dopamine levels, whereas Ag NPs were moderately effective in changing gene expression.[166] Braydich-Stolle et al.[107] studied 15 nm Ag NPs, 30 nm MoO_3 NPs, and 30 nm Al NPs along with their bulk counterparts in mouse spermatogonial stem cells at 5, 10, 25, 50, and 100 μg/mL concentrations after 48 h. They found that the small (~15 nm) Ag NPs were more toxic than similar-sized Al or MoO_3 NPs. In studies by Soto et al.[162,163,165] with murine alveolar macrophage cell lines, human macrophages, and epithelial lung cell lines exposed to Ag, TiO_2, Fe_2O_3, Al_2O_3, ZrO_2, and Si_3N_4 NPs, they found that Ag NPs displayed the greatest relative toxicity at multiple concentrations and in the different cell lines compared to moderate toxicity for Fe_2O_3, Al_2O_3, ZrO_2 and anatase TiO_2 and low toxicity for rutile TiO_2 and Si_3N_4.[163–165] In animal studies, the ranking of toxicity has been demonstrated as Ag > Cu > Al.[97,169]

Many of the previous *in vitro* studies demonstrated high toxicity for Zn and Ag-based NP compositions[127,157] and low toxicity for Fe_3O_4 NPs. This trend is further supported by other comparative *in vitro* studies demonstrating the lower toxic potential of Fe_2O_3 compared to $VOSO_4$, TiO_2, SiO_2, and NiO at concentrations from 1 to 100 μg/mL after 24 h[167] or Mn_3O_4 or Co_3O_4 at concentrations of 30 μg/mL after 4 h.[168] In contrast, an animal study with ferric oxide NPs (Fe_2O_3) (22 and 280 nm) found oxidative stress in the lungs of rats intratracheally exposed to low (0.8 mg/kg) and high (20 mg/kg) doses for 1, 7, and 30 days.[170]

The above results, summarized in Tables 2 and 3, clearly indicate that NPs should not be viewed as a homogenous population with simple toxic attributes, but rather that NPs act independently to mediate biological reactions. In general, Zn-based NPs are more toxic than Ag or Cu NPs, which are more toxic than most other metal NP compositions. However, it should be mentioned here that not all NPs show toxicity and in some cases NPs such as CeO_2can have positive effects such as the suppression of ROS production.[158] Furthermore, the majority of these studies are performed *in vitro* and there is very little evidence that these toxicity rankings directly translate into *in vivo* systems, which will be further elaborated upon in the following section.

CONCLUSIONS, CHALLENGES, AND FUTURE OUTLOOK

In summary, these studies indicate that although NPs have far-reaching applications, they also have the potential to cause adverse effects at the cellular, subcellular, and protein levels (Tables 1–3). The basis of the undesirable effects of NPs may stem from their small size (surface area to volume ratio and size distribution), chemical composition (purity, crystallinity, electronic properties, etc.), surface

TABLE 2 | Selected Comparative *In Vitro* Toxicity Studies

Nanoparticle Rank for Toxicity	Cell line(s)	Dose, Time	Comments	References
Cu>Zn>Co>Sb>Ag>Ni>Fe >Zr>Al_2O_3> TiO_2>CeO, low toxicity for W	Two human pulmonary cell lines (A549 and THP-1)	0.1–3300 μg/mL, 3 and 24 h	MTT assay on THP-1 cell line exposed to NP for 24 h most sensitive experimetnal design	Lanone et al.[92]
ZnO>CeO_2/TiO_2	BEAS-2B	6.125–50 μg/mL, 1–6 h	ZnO comparatively more toxic than TiO_2 or CeO2 due to particle dissolution to Zn^{2+}	George et al.[158]
ZnO>CeO_2/TiO_2	BEAS-2B and RAW264.7 macrophages	10–50 μg/mL, 1–24 h	ZnO dissolution in endosomes, CeO_2 suppressed ROS production, TiO_2did not elicit protective or adverse effects	Xia et al.[159]
ZnO>Fe_2O_3>TiO_2/CeO_2	Human mesothelioma and rodent fibroblast cell line	30 μg/ml, 3–6 days	Human MSTO cells highly sensitive to Fe_2O_3	Brunner et al.[160]
ZnO>Fe>SiO_2	L2 rat epithelial cells, rat primary alveolar macrophages and co-cultures	0.0052–520 mg/cm^2, 1–48 h	*In vivo* and *in vitro* measurments demonstrated little correlation	Sayes et al.[161]
ZnO>TiO_2, Fe_3O_4, Al_2O_3 and CrO_3	Neuro-2A cell line	10–200 μg/mL, 2–72 h	ZnO was more toxic compared to other NPs	Jeng and Swanson[157]
$CdCl_2$>$CdSO_4$>$ZnSO_4$>ZnO> $CuSO_4$>$ZnCl_2$>V_2O_5, >$CuCl_2$ >$NiSO_4$>$NiCl_2$>$Fe_2(SO_4)_3$> $CrCl_2$>VCl_2>$CrK(SO_4)_2$>$FeCl_2$	A549	0.005–5 mM, 2–48 h	RLE-6TN rat epithelia cells more sensitive than A549 cells	Riley et al.[162]
Ag>Fe_2O_3>Al_2O_3>ZrO_2> Si_3N_4>TiO_2 in RAW264.7, ZrO_2>Al_2O_3/Fe_2O_3/Si_3N_4/Ag> TiO_2 in THB-1 and A549	Murine alveolar macrophage (RAW264.7), human macrophage (THB-1), and human epithelial A549	5 μg/mL, 48 h	THB-1 and A549 cells more sensitive than RAW264.7, no correlation between specific surface area or NP morphology to toxicity	Soto et al.[163,164]
Ag>MoO_3>Al/Fe_3O_4/TiO_2	Rat cell line (BRL 3A)	5–25 μg/mL, 24 h	Ag produces toxicity through oxidative stress	Hussain et al.[127]
Ag>Mn	PC-12 cells	1–100 μg/mL, 24 h	Ag produced cell shrinkage and irregular membrane borders, Mn dose dependently depleted dopamine	Hussain et al.[126]
Ag>NiO>TiO_2	Murine macrophage cell line	5 μg/mL, 48 h	Nanoparticles characterized as aggregates, caution on Ag	Soto et al.[165]
Ag>MoO_3>Al	Mouse spermatogonial stem cells	5–100 μg/mL, 48 h	Concentration-dependent toxicity for all NPs tested	Braydich-Stolle et al.[107]
Cu, Mn>Al	PC-12 cells	10 μg/mL, 24 h	Txnrd1, Gpx1, Th, Maoa, Park2, Snca genes expression altered	Wang et al.[166]
$VOSO_4$>TiO_2,SiO_2,NiO,Fe_2O_3,CeO_2,Al_2O_3	BEAS-2B	1–100 μg/mL, 24 h	Manufactured pure oxides less toxic than natural particulate matter derived from soil dust, IL-6 secretion did not correlate with the generation of ROS in cell-free media	Veranth et al.[167]
Mn_3O_4>Co_3O_4>Fe_2O_3>TiO_2	Lung epithelial cells A549	30 μg/mL, 4 h	Acellular ROS assay demonstrates catalytic conditions of NPs based on elemental composition	Limbach et al.[168]
Al>Al_2O_3	Rat alveolar macrophages	25–250 μg/mL, 24 h	Phagocytosis hindered after exposure to Al NPs	Wagner et al.[108]

TABLE 3 | Selected Comparative *In Vivo* Toxicity Studies

Nanoparticle(s)	Animal	Dose/Route	Result	References
Ag	Rat	30–1000 mg/kg (sub acute oral for 28 days)	Dose-dependent effect on alkaline phosphatase and cholesterol. Twofold more accumulation of NP in kidneys of female than male	Kim et al.[130]
Ag	Rat	1.73×10^4/cm^3 to 1.32×10^6/cm^3 (sub acute inhalation, 6 h/day, 5 days/week for 4 weeks)	Liver histopathological effect, but no effect in hematology and biochemical parameters	Ji et al.[131]
Ag	Zebrafish	5–100 μg/mL (exposure, 72 h)	Dose-dependent toxicity in embryos. Ag NP distributed in brain, heart, yolk, and blood of embryos	Asharani et al.[132]
Ag	Rat	NP was implanted intromusculary for 7, 14, 30, 90, and 180 days	Inflammation	Chen et al.[101]
Ag	Mice	100–1000 mg/kg (acute oral)	Oxidative stress gene expression alterations	Rahman et al.[133]
Ag, Cu, and Al	Mice and Rat	30–50 mg/kg (intravenous/ intraperitoneal)	**BBB** penetration	Sharma[169]
Au	Mice	2×10^5 PPB (oral for 7 days)	NP uptake occurred in the small intestine by persorption through single, degrading enterocytes extruded from a villus. Smaller particles cross the GI tract more readily	Hillyer et al.[115]
Cu	Zebrafish	0.25–1.5 mg/L (exposure, 48 h)	Biochemical, histopathological changes, and alterations in gene expression	Griffitt et al.[138]
Cu	Mice	108–1080 mg/kg (acute oral)	NP-induced gravely toxicological effects and heavy injuries on kidney, liver, and spleen of treated mice	Chen et al.[135]
Fe_2O_3	Rat	0.8–20 mg/kg (inhalation)	Oxidative stress, inflammation, and pathology	Zhu et al.[170]
TiO_2	Mice	5 g/kg (acute oral)	Biochemical and histopathological effects	Wang et al.[148]
SiO_2 Magnetic-NPs	Mice	25–100 mg/kg (intraperitoneal for 4 weeks)	NPs were detected in brain indicating BBB penetration	Kim et al.[84]

charge, surface structure (surface reactivity, surface groups, inorganic, or organic coatings), solubility, shape, and aggregation behavior.[8] Furthermore, NPs can cause behavioral, physiological, and metabolic alterations in exposed animals. To provide some general conclusions, this section will address some of the critical parameters linked to NP toxicity and hurdles to overcome in understanding the potential toxicity of NPs.

Revisiting the Definition of Toxicity: Dose, Time, and Route of Administration

The toxicity of a chemical depends on the dose (acute/sub acute/chronic), time of exposure (short or long term), and route of administration (inhalation/oral/dermal). Subsequently, even seemingly nontoxic materials such as water, if taken in larger quantities, will flush out salts from the body and result in toxicity. Therefore, the doses selected for toxicity studies typically represent a range where there are minimal effects up to concentrations where toxicity becomes apparent. However, these ranges of doses and exposure methods may not represent realistic NP exposure conditions or relevant biomedical dosages. For example, Wang et al.[146,154] orally administered large doses of 5 g TiO_2 NPs or Zn NPs per kilogram of body weight to mice.[148,156] The purpose of the above studies was to evaluate the oral toxicity of nanoscale TiO_2/Zn according to the OECD guidelines, which

are currently used for testing the toxicity of chemicals. Therefore, the doses used in these studies were higher than the possible exposure level. Fortunately, the ability to administer a wide range of doses in various manners provides threshold values for toxicity and potentially novel interactions between cells and NPs.

Current Opinion on Toxicity Models: Cells versus Animals

One limitation of most toxicity studies thus far is that these results have mainly come from using *in vitro* cell culture models and there has been little correlation between *in vivo* and *in vitro* measurements.[161] Therefore, further *in vivo* studies should be conducted to more fully characterize and understand the biodistribution and potential adverse responses of NPs. Furthermore, target organs should be identified to improve *in vitro* cell culture models and draw relevant conclusions to organ-specific NP toxicity in animal studies. For example, contrasting conclusions were drawn from the studies of different organs (i.e., liver vs oral, dermal, pulmonary, and genotoxicity studies in different animals).[148,171] Wang et al.[148] reported biochemical alterations along with pathology of the liver and nephrotoxicity in female mice dosed with TiO_2 NP-treated, whereas Warheit et al.[171] found very low oral toxicity, no skin irritation, low inflammatory potentia, and no genotoxic effects of ultrafine TiO_2 (>100 nm) NPs.

To further compound the existing toxicity data, various cell types have shown sensitivity to certain NP compositions. For example, Schrand et al.[81] demonstrated that alveolar macrophages were more sensitive to carbon-based NPs compared to neuroblastoma cells. Lanone et al.[92] proposed that the MTT assay on THP-1 cells at 24 h was a more sensitive assay for toxicity compared to A549 cells or a 3-h time point, whereas Brunner et al.[160] found that human mesothelioma cells were more sensitive to Fe_2O_3 than a rodent fibroblast cell line. Riley et al.[162] found that RLE-6TN rat epithelia cells were more sensitive than A549 cells, and Soto et al.[164] found that THB-1 and A549 cells were more sensitive than RAW264.7 cells (Table 2).

The indirect versus direct effects of NPs have recently been explored.[172] They found that cobalt–chromium (CoCr) NPs indirectly exposed to human fibroblasts through a multi-layered barrier of confluent BeWo cells experienced similar DNA damage after 24 h, compared to direct NP exposure. Although there was evidence for CoCr NP uptake in the uppermost cells of the BeWo cell layer, there was no proof of NP translocation across this multi-layered membrane to the underlying fibroblasts, suggesting that NP uptake does not have to occur for cellular damage. Furthermore, the dissolution of the CoCr NPs into ions, which were able to transverse the BeWo cell layers and reach the fibroblasts, may not be directly responsible for the DNA damage because direct exposure of fibroblasts to Co(II) at 20 ppb did not cause DNA damage. The authors propose that signals generated within the barrier involving connexin or pannexin channels associated with ATP release contribute to DNA damage and demonstrated that blockers of these channels can decrease DNA damage after indirect exposure to CoCr NPs. Similarly, Ag NPs can produce mitochondrial toxicity without toxicity in human fibroblasts.[173] Therefore, the indirect effects and subcellular organelle damage should be further scrutinized during future toxicity research.

Although it has been reported that Ag NPs localize to critical organelles such as the mitochondria and nuclei,[173] the reliance on TEM images alone is not sufficient and should be further substantiated by overlaying fluorescent images of stained mitochondria and NPs[174] or with differential separation techniques[175] to verify NP localization. Many other studies are beginning to elaborate on the mechanisms of uptake. For example, Chithrani et al.[174] reported cellular uptake and transport of Au NPs in breast cancer cells (MCF-7).[176] They found that particles were first internalized through receptor-mediated endocytosis and trapped in endosomes; these endosomes then fused with lysosomes for processing before being transported to the cell periphery for excretion. In a different study, the accumulation of TiO_2 NPs in the cytoplasm of Chinese hamster ovary cells was demonstrated after exposure to 10, 100, 300, and 1000 μg/mL doses without entry into the nucleus in a dose, time, and size-dependent manner.[177] Although it is currently unknown if there is a direct correlation between NP uptake and toxicity, several studies have shown that NP uptake can reduce cellular functions such as phagocytosis in macrophages.[108,178]

Proper NP Characterization and Assessment of NPs in the Biological Milieu

There have been a number of studies suggesting characterization techniques prior to, during, and after toxicity studies[7,10,11] should be addressed. For example, Powers et al.[11] suggested techniques such as DLS, centrifugal sedimentation, laser diffraction/static light scattering, low pressure impactor, size exclusion chromatography, electron microscopy, time-of-flight mass spectroscopy, and atomic force microscopy to determine NP size. However, the burden of testing

many or all of these parameters typically requires large quantities of sample, a variety of scientific equipment, expertise, time, and resources. In an effort to simplify the process, certain characteristics have been identified as priorities including composition, size, shape, dispersion, physical and chemical properties, surface area, and surface chemistry.[6–8,11,177] Measuring the NPs in a dry state may initially provide some information, but does not represent the interface once dispersed in biological media or the body.

The change in size due to NP agglomeration raises concerns about the validity of studies where NP size effects have been implicated. For example, NP agglomeration in biological fluids can alter delivery kinetics and dosing parameters.[178] Furthermore, if chemicals or surface modifications are used to alter the dispersion, size may only be one factor between well-dispersed and agglomerated NPs often confounded with the surface charge/chemistry of the NP. Other factors shown to alter NP properties include duration of suspension in aqueous solutions, dissolution, and oxidation. For example, the analysis of Cu NPs in water over a 34-day time period demonstrated increased NP agglomeration and fluctuating zeta potentials.[179] The Cu NPs also displayed altered morphologies under TEM imaging after the 30-day time period changing from spherical in nature to a more crystalline form with spikes emanating from the NP surface. Other studies examining NP properties with TEM have also demonstrated the aggregation and internalization of manufactured NPs including Ag.[163] However, it is a worthwhile goal to examine size-dependent toxic effects with NPs that display more uniform dispersions such as SiO_2 NPs,[121] where the size element and surface area components of NPs can be better correlated to toxicity measurements.[180,181] The size effects of NPs that aggregate in solution can be appropriately assessed through multiple characterization techniques, which will be critical before size-dependent toxicity claims can be confirmed.

Specific Physicochemical Properties Linked to NP Toxicity

The absorption, distribution, metabolism, excretion, and toxicity of NPs are largely dependent on their physicochemical properties and the surrounding environmental conditions,[182] which will be considered in the following section.

Most *in vitro* and *in vivo* studies have shown a high response to Zn, Cu, Ag, and Ni NPs compared to other elemental compositions such as MoO_3, Al, Fe_3O_4, TiO_2, and CeO NPs (Table 1). The finding that Ag NPs show a great toxic response to cells and animals may seem surprising because Ag metal is nontoxic to humans and animals in its bulk chemical form.[54] With other NP compositions such as ZnO, dissolution to Zn^{2+} has been implicated in its strong toxicity.[158,159] Similarly, in animal studies Ag and Cu demonstrated greater neurotoxic effects than Al NPs.[97] Assessing the cellular ROS production of metal NPs has led to a greater understanding of the relationship between elemental composition, catalytic potential, and toxicity.[168] However, correlating the generation of ROS in cell-free media may not match with other toxicity end points such as cytokine (IL-6) secretion[167] and will need to undergo further scrutiny before becoming accepted as a standard characterization technique.

As the size of a particle decreases, its surface area to volume ratio increases, allowing a greater proportion of its atoms or molecules to be displayed on the surface resulting in increased surface reactivity.[8] Oberdoerster et al.[7] reported that particles with greater specific surface area per mass were more biologically active and that their biological effects mainly depended on their surface area rather than particle mass.[183] As particle size shrinks, there is a tendency for toxicity to increase, even if the same material is relatively inert in a bulk form.[154] For example, Yu et al.[121] demonstrated that smaller SiO_2 NPs, with a higher specific surface area, produced more toxic effects compared to larger-sized NPs. Several *in vitro* and *in vivo* studies with Au NPs have demonstrated that smaller NPs are more toxic than larger NPs[109,184] and can induce immunological responses such as the up-regulation of pro-inflammatory genes IL-1, IL-6, and TNF-α.[110] Also, studies with TiO_2 NPs suggest that smaller NPs are more toxic.[139,143] Most of the effects of shape have been studied in Au NPs, which demonstrate that Au rods typically display greater toxicity than Au spheres.[111,185–187] However, the biocidal activity of truncated triangular Ag nanoplates was greater than spherical and rod-shaped Ag NPs against *E. coli*.[188]

NP surface chemistry and surface charge play important roles in toxicity and corresponding safety assessments.[189] Altering the surface chemistry of NPs has been shown to effectively prevent toxicity derived from the core material.[28,190–192] For example, polysaccharide coatings have been used to promote biocompatibility as well as better dispersion in solution.[128,193] With regard to surface charge, positively charged Au NPs caused greater toxicity than those with negative surface charges.[112] Al_2O_3 NPs were less toxic than metallic Al NPs suggesting that surface oxide formation may also

alter bio-interactions.[108] Studies with Cu NPs have demonstrated time-dependent surface oxide formation that can be monitored through changes in the surface morphology and zeta potential (charge) of the NPs, although its link to toxicity is not fully understood.[179] Furthermore, the crystal structure of the NP can dictate its toxic potential, with the different forms (i.e., rutile, anatase, and amorphous) of TiO_2 NPs being a prime example.[145] Although the mechanisms of surface chemistry, surface charge, and crystallinity-based toxicity are complex, studies are beginning to elucidate certain surface functional groups and properties that can effectively alter biological responses.

Mechanisms of NP-induced Toxicity and Other Bio-effects

NPs with their small size and large surface area have been reported to interact with proteins[182] and enzymes within mammalian cells and to generate ROS. When the depletion of the antioxidant defense mechanism occurs and ROS accumulate, an inflammatory response can be initiated leading to the perturbation and destruction of the mitochondria resulting in eventual programmed cell death.[50] Other cellular level changes associated with current mechanisms of toxicity include decreases in GSH levels and an up-regulation of oxidative stress and inflammatory genes. Several studies with Ag NPs have demonstrated toxicity through an oxidative stress pathway.[127,128]

Although some NPs may appear to be nontoxic, other cellular mechanisms such as cell signaling and other normal cellular functions may be disrupted and are currently undergoing further investigation. For example, very small (~4–5 nm) nanodiamonds, various functionalized carbon nanotubes, and cerium NPs have not shown any obvious toxic effects to cells in culture.[101,194–199] In contrast, substantial biochemical changes such as dopamine depletion have been observed after exposure to Mn or Cu NPs.[111,126] Therefore, current studies are addressing how to better define the interactions of cells with NPs of different compositions, sizes, and surface chemistries at the molecular level as well as establishing databases to define and predict NP toxicity. Although few databases are available, Nanowerk[200] has established a database of 2341 NPs from 152 suppliers, whereas the Nano Health Environment Commented Database project[201] is working to create a critical and commented database on the health, safety, and environmental impact of NPs. Through these integrated efforts, the potential risks and benefits of NPs are sure to be realized.

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