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Adv Exp Med Biol. Author manuscript; available in PMC 2016 March 21.

Published in final edited form as:

Adv Exp Med Biol. 2014 ; 811: 111–134. doi:10.1007/978-94-017-8739-0_7.

Intracellular Signal Modulation by Nanomaterials

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Abstract

A thorough understanding of the interactions of nanomaterials with biological systems and the resulting activation of signal transduction pathways is essential for the development of safe and consumer friendly nanotechnology. Here we present an overview of signaling pathways induced by nanomaterial exposures and describe the possible correlation of their physicochemical characteristics with biological outcomes. In addition to the hierarchical oxidative stress model and a review of the intrinsic and cell-mediated mechanisms of reactive Oxygen species (ROS) generating capacities of nanomaterials, we also discuss other oxidative stress dependent and independent cellular signaling pathways. Induction of the inflammasome, calcium signaling, and endoplasmic reticulum stress are reviewed. Furthermore, the uptake mechanisms can crucially affect the cytotoxicity of nanomaterials and membrane-dependent signaling pathways can be responsible for cellular effects of nanomaterials. Epigenetic regulation by nanomaterials effects of nanoparticle-protein interactions on cell signaling pathways, and the induction of various cell death modalities by nanomaterials are described. We describe the common trigger mechanisms shared by various nanomaterials to induce cell death pathways and describe the interplay of different modalities in orchestrating the final outcome after nanomaterial exposures. A better understanding of signal modulations induced by nanomaterials is not only essential for the synthesis and design of safer nanomaterials but will also help to discover potential nanomedical applications of these materials. Several biomedical applications based on the different signaling pathways induced by nanomaterials are already proposed and will certainly gain a great deal of attraction in the near future.

Keywords

Signaling; Nanotoxicology; Nanomedicine; Oxidative stress; Protein interaction; Cell death; Physico-chemical characteristics

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7.1 Introduction

The increasing utilization of nanomaterials in consumer products, environmental sectors and nanomedicine has led to increased consumer, occupational and environmental exposure to these materials. The study of possible adverse health effects is thus warranted for the development of safe and consumer friendly nanotechnology. Many nanomaterials have indeed been shown to be toxic *in vitro* and *in vivo*, inducing inflammation, genotoxicity and cell death in various organ systems. Understanding the underlying cellular and molecular mechanisms is of crucial importance as we aim to design safer nanomaterials. Shvedova and colleagues have proposed that nanotoxicology must be defined as a discipline studying the interference of engineered nanomaterials with the function of cellular and extracellular nanomachineries (Shvedova et al. 2010). This definition emphasizes not only the description of specific responses that are directly related to the nanomaterial size, but also the understanding of the underlying mechanisms. Furthermore it is important to analyze the consequences of nanomaterial-protein interactions on signal transduction. Finally, the establishment of common features of nanomaterials, which are responsible for the observed effects, is essential to predict possible adverse health effects of new materials. A better understanding of signal modulations induced by nanomaterials will also help to discover potential biomedical applications of these materials.

This chapter reviews the current understanding of cellular effects (anti-oxidant defense, inflammation, apoptosis) induced by nanoparticles (NP) with focus on the underlying mechanisms and signal modulations. We discuss oxidative stress dependent mechanisms as well as signaling pathways, which are independent of reactive oxygen species (ROS) production. We emphasize the role of NP-protein interactions in the effects induced and describe the physico-chemical characteristics of nanomaterials responsible for the cellular effects.

7.2 Hierarchical oxidative stress model

One of the major biological effects of nanomaterials is the production of ROS within the cells. Controlling the level of the cellular redox status is crucial for normal cell function and is a finely regulated process. The production of cellular oxidants is counterbalanced by the presence of anti-oxidants which allow maintaining a low level of ROS to prevent cell damage. An imbalance of this equilibrium leads to oxidative stress which may result at sustained high levels in the oxidative attack of cellular molecules including membrane constituents, proteins and the genomic DNA ultimately leading to cell death. On the other hand, during a transient induction of oxidative stress, ROS can act as second messengers in redox sensitive signaling pathways through reversible and transient protein oxidations, regulating their activity. Indeed, ROS can activate transcription factors such as NF-κB (nuclear factor-kappa B) which induces the expression of genes involved in pro-inflammatory responses and apoptosis whereas activation of the transcription factor AP-1 (activator protein 1) by ROS leads to proliferation and differentiation. At lower levels of oxidative stress ROS induce the antioxidant defence by activating the nuclear factor (erythroid-derived-2)-related factor 2 (Nrf-2). This transcription factor binds to the antioxidant responsive elements (ARE) to induce the expression of phase II detoxifying and

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antioxidant enzymes such as glutathion *S* transferase (GST), γ glutamyl cysteine synthetase (GCS), nicotinamide adenine dinucleotide phosphate quinone oxidoreductase (NQO1), and heme oxygenase-1 (HO-1). A hierarchical cellular response to oxidative stress is thus observed, inducing an anti-oxidant defense at low levels, pro-inflammatory responses and proliferation at higher levels and finally cell death at very high oxidative stress levels. This three tiered oxidative stress model was proposed by Nel et al. to account for the toxicity of nanomaterials (Nel et al., 2006). Several studies have since confirmed the central role of ROS production in the toxicity of numerous nanomaterials.

7.2.1 Mechanisms of reactive oxygen species production by nanomaterials

Nanomaterials can generate and induce the production of ROS through different mechanisms (Figure 7.1). The nanomaterial surface could present surface bound radicals such as $O_2^{\circ -}$, OH°, SiO° or TiO° which may react with O_2 to form $O_2^{\circ -}$ radicals which in turn could generate other ROS. Structural defects on the particle surface could also lead to the formation of reactive groups. Finally, the nanomaterial surface may also include transition metals which could generate ROS through Fenton-type and Haber-Weiss-type reactions. Furthermore, environmental oxidants such as ozone, semiquinones and NO could adsorb onto the nanomaterial surface and enter cells through the so called “Trojan horse effect”. In addition to these inherent ROS generating properties, nanomaterials could also indirectly enable ROS production by triggering cellular mechanisms. Damage or activation of mitochondria could lead to the release of ROS produced by the mitochondrial electron transport chain. Another source of intracellular ROS is the nicotinamide adenine dinucleotide phosphate (NADPH) oxidase which could be activated by nanomaterials as shown for ZnO NPs (Wilhelmi et al., 2013). This membrane bound enzyme is highly expressed in neutrophils and macrophages to ensure the respiratory burst for killing invading microorganisms through ROS production. Under physiological conditions this enzyme complex is latent in phagocytic cells. However, nanomaterials can activate the inflammatory cells inducing a respiratory burst in the absence of bacteria (Abrikossova et al., 2012, Tulinska et al., 2013). NADPH oxidase is abundant in “professional” phagocytes but this protein is also present in non inflammatory cells where it contributes to cell signaling. Involvement of NADPH oxidase in CeO_2 and CoCr NP toxicity has for instance been demonstrated in fibroblasts (Culcasi et al., 2012, Raghunathan et al., 2013). Other enzymes also generate ROS as by-products of their activity such as cytochrome P450, xanthine oxidase, lipoxygenase, cyclooxygenase as well as enzymes within the peroxisome complex. Activation of macrophages is an especially important mechanism of ROS production by high aspect ratio nanomaterials (HARN) as long, thin and biopersistent fibres could lead to “frustrated phagocytosis”. This mechanism leads to the persistent release of oxidants and pro-inflammatory mediators and has been firstly described to account for the toxicity of asbestos but it has since been observed also for carbon nanotubes (CNT) (Murphy et al., 2012). A further indirect mechanism of oxidative stress induction by nanomaterials is the depletion or inhibition of anti-oxidants leading to an imbalance of the redox homeostasis of the cell. Interference of nanomaterials with the scavenging properties of anti-oxidants and metalloproteins or inhibition of the synthesis of enzymatic or non enzymatic anti-oxidants by nanomaterials will indirectly trigger oxidative stress. For instance, Pl/Au nanorods have been shown to reduce the ability of ascorbic acid to scavenge radicals (Zhou et al., 2013).

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Nanomaterials may also inhibit repair mechanisms which eliminate molecules damaged by ROS potentiating their toxicity. On the other hand, some nanomaterials could also scavenge radicals on their surface or exert anti-oxidant properties (e.g. Au, Ag, CeO_2 and Pl NPs). CeO_2 NPs have oxygen buffering capacities which are attributed to the valence state of cerium and to defects in the crystal structure which are increased at the nano-scale. The anti-oxidant properties of CeO_2 NPs thus depend on the nanocrystal diameter (Lee et al., 2013) and the ratio of surface Ce^{3+}/Ce^{4+} (Das et al., 2012). This interesting anti-oxidant property of some nanomaterials is intended to be used in nanomedical applications (Colon et al., 2010, Rehman et al., 2012).

There are thus at least two major mechanisms of oxidative stress induction by nanomaterials. The physico-chemical properties of nanomaterials could generate ROS in acellular conditions based on the intrinsic oxidant potential of the particles. Overall, nanomaterials with or without such oxidative properties may however also produce oxidants *via* cell-mediated mechanisms. It has for instance been shown that TiO_2 NPs have a low intrinsic capacity to produce oxidants compared to carbon black (CB) NPs but have been shown to induce the same level of intracellular ROS in bronchial epithelial cells (Hussain et al., 2009) this implies that the oxidative stress potential of nanomaterials cannot solely be deduced by their cell-free oxidant generating properties.

7.2.2 Paradigm of the graduated oxidative stress responses induced by nanomaterials

ROS production induced by nanomaterials has been shown to orchestrate various cellular effects through different signaling pathways dependent on the level of oxidative stress induced. Many nanomaterials (CNT (Brown et al., 2010), Ag (Kang et al., 2012) (Prasad et al., 2013), SiO_2 (Gehrke et al., 2013), CuO (Piret et al., 2012), and tungsten carbide Co (Zhang et al., 2010) have been shown to induce the activation of the transcription factor Nrf-2 leading to an increase of the expression of phase II detoxifying as well as antioxidant enzymes *in vitro*. The involvement of Nrf-2 in inducing an anti-oxidant defense to protect cells against nanomaterials was confirmed using Nrf-2 knockdown cells (Piret et al., 2012). Activation of this signaling pathway and antioxidant defense has also been demonstrated *in vivo* for TiO_2 NPs (Ze et al., 2013). ROS-dependent inflammatory responses are also very common nanomaterial effects induced through MAPK signaling and activation of NF-κB dependent inflammatory cytokine gene expression and have recently been reviewed. (Manke et al., 2013). The activation of the transcription factor AP-1 by nanomaterials has been reported less frequently but it has been shown to be induced *in vitro* and *in vivo* by CuO (Piret et al., 2012), CB (Mroz et al., 2007), CNT (Pacurari et al., 2008), Co (Wan et al., 2008), WC-Co (Ding et al., 2009), Al_2O_3 (Dey et al., 2008) and iron oxide NPs (Murray et al., 2012). High levels of oxidative stress may finally lead to cell death and nanomaterials have been shown to induce different apoptotic or necrotic signaling pathways which are discussed in section 7.4.

Nanomaterials have thus been shown to induce different ROS dependent signaling pathways in accordance with the oxidative stress paradigm proposed by Nel et al. (Nel et al., 2006). Activation of these signaling pathways by nanomaterials may thus induce an anti-oxidant defense at low levels of oxidative stress, proliferation and pro-inflammatory responses at

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higher oxidative stress levels and finally cell death at deleterious levels of ROS. High throughput analysis of signaling pathways, protein and gene expression have confirmed the induction of these cellular responses *in vitro* as well as *in vivo*. Gene expression profiles have shown that nanomaterials induce mostly expression of mRNAs associated with cell signaling, metabolism, and stress, but also cytoskeleton and vesicle trafficking or cell membrane proteins. Depending on the nanomaterials and concentrations the induced mRNAs or proteins were related to inflammation, cell cycle, apoptosis or DNA repair (Perkins et al., 2012), (Yang et al., 2010), (Witzmann and Monteiro-Riviere, 2006), (Busch et al., 2010), (Ganguly et al., 2009), (Qu et al., 2013b), (Ze et al., 2013), (Okoturo-Evans et al., 2013). High throughput screening of signaling pathways induced by several metal oxide NPs in macrophages have been performed and the use of Self Organizing Map (SOM) analysis revealed two cluster groups of sub-lethal pro-inflammatory responses and of lethal genotoxic responses (Rallo et al., 2011) confirming the hierarchical cellular response to NPs. These approaches may also allow developing quantitative structure-activity relationships (QSAR). Some papers have compared the protein expression, gene expression and protein phosphorylation within the same study such as Ge and colleagues allowing the establishment of protein-interacting networks and upstream signaling pathways of toxicity responses but also detoxification pathways of TiO_2 in bronchial epithelial cells (Ge et al., 2011). These cellular effects may have several pathophysiological consequences such as inflammation or fibrosis.

7.3 Other cellular mechanisms induced by nanomaterials

Beyond this graduated oxidative stress response paradigm other mechanisms could also induce cellular responses. Some of these effects are directly or indirectly linked to oxidative stress, but others are ROS-independent mechanisms. These mechanisms could however sometimes lead to intracellular ROS production and it is thesefore often not easy to distinguish whether ROS are the cause or the consequence of cellular signaling pathways.

7.3.1 Oxidation of biomolecules

The induction of high levels of oxidative stress by nanomaterials can lead to oxidation of cellular molecules. Oxidative DNA damage has been evidenced for many nanomaterials using several techniques such as the Fpg (formamidopyrimidine-DNA glycosylase) modified comet assay or detection of 8-hydroxydeoxyguanosine by immunohistochemistry or HPLC. This genotoxic insult can either be repaired or induce apoptosis but failure of these protection mechanisms could lead to mutagenesis. Protein oxidation could also be a consequence of oxidative stress which could lead to their inactivation as well as activation. The redox proteome (reversible and irreversible covalent protein modifications) links redox metabolism to biological structure and function. Lipid peroxidation on the other hand can induce lysosomal damage and this mechanism can be responsible for TiO_2 induced apoptosis (Hussain et al., 2010) and can lead to inflammasome activation.

7.3.2 Inflammasome activation

Inflammatory responses could not only be induced by NF-κB signaling but also through activation of the inflammasome. This multiprotein complex promotes the maturation of

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proinflammatory cytokines IL-1 beta and IL-18 and could be activated by ROS but also by other cellular mechanisms. For instance cathepsin B, which is released after lysosome rupture induced by ROS dependent as well as ROS independent mechanisms can activate the inflammasome. A cathepsin B dependent activation of the inflammasome has been shown to be responsible for TiO_2 induced production of IL-1beta by macrophages (Morishige et al., 2010). TiO_2 NPs have also been shown to induce lipid peroxidation leading to lysosomal destabilisation with subsequent release of cathepsin B in bronchial epithelial cells (Hussain et al., 2010). Conversely, amino-functionalized polystyrene NPs induced lysosomal destabilisation through a ROS independent mechanism due to proton accumulation within the lysosomes of macrophages due to the so-called "proton sponge" effect of these NPs (Lunov et al., 2011). Interestingly, iron oxide NPs induce lysosomal permeability but could suppress the lipopolysacchrides (LPS) induced production of IL-1 beta by decreasing the activity of cathepsin (Wu et al., 2013). This inhibitory effect was however not observed for SiO_2 or TiO_2 NPs which increased the LPS induced inflammation through activation of the inflammasome (Winter et al., 2011, Sandberg et al., 2012). Mano et al. have also shown that TiO_2 NPs induce inflammatory responses by interaction of the NPs with Toll Like receptor 4 (TLR4) which is known to induce the inflammasome (Mano et al., 2013). The cell death receptor P2X7 could also induce inflammasome activation through efflux of K^+ and SiO_2 and TiO_2 NPs have been shown to activate P2X7 leading to IL-1 beta secretion (Dekali et al., 2013). Others have also shown that NLRP3 inflammasome activation by TiO_2 involves K^+ efflux but did not require NP uptake leading to neutrophil recruitment *in vivo* through IL-1 alpha secretion (Yazdi et al., 2010). The activation of the inflammasome could not only lead to inflammation but also to cell death through pyroptosis as observed for CB NPs (Reisetter et al., 2011) or induction of matrix metalloprotease 1 as shown for TiO_2 NPs in pulmonary fibroblasts (Armand et al., 2013). Recent studies have shown that in synergy with toll-like receptor ligands, certain CB NPs could promote the activation of the NLRP3 inflammasome. This activation was shown to depend on the chemical surface functionalization (Yang et al., 2013). However, it remains to be clearly determined which CB NP-dependent signals trigger this activation.

7.3.3 Calcium signaling

Nanomaterials could also induce calcium signaling by increasing intracellular concentrations of Ca^{2+}. TiO_2 NPs have for instance been shown to induce the opening of L-type Ca^{2+} channels on mast cells as well as non specific influx of extracellular Ca^{2+} by permeation of the plasma membrane through an oxidative stress dependent mechanism (Chen et al., 2012b). A sustained elevation of Ca^{2+} was achieved by inducing the release of Ca^{2+} from the endoplasmic reticulum leading to histamine secretion. TiO_2 NPs also stimulate mucin secretion in epithelial cells by inducing extracellular Ca^{2+} influx and calcium release from the endoplasmic reticulum (Chen et al., 2011). Intracellular Ca^{2+} increase is also involved in the inhibition of cell proliferation by Ag NPs (Asharani et al., 2009).

7.3.4 Endoplasmic reticulum stress

Induction of endoplasmic reticulum stress and signaling by certain nanomaterials has recently attracted interest (Zhang et al. 2012). The endoplasmic reticulum fulfills multiple cellular functions and many disturbances cause accumulation of unfolded proteins in the

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endoplasmic reticulum, triggering an evolutionarily conserved response, termed the unfolded protein response (UPR). The UPR is a signaling pathway, which is activated to regulate protein synthesis and restore normal equilibrium, in case of increased protein load or accumulation of unfolded or malformed proteins. The UPR leads to decreased protein synthesis and production of chaperons do facilitate protein folding. Once activated, UPR can either result in recovery or activate a cascade of reactions leading to inflammation through NF-κB and proeasome activation or ultimately to apoptosis. In a recent study, Christen and Fent have shown that Ag and silica NPs could induce the UPR pathway. Interestingly, induction of ER stress was not directly linked to the formation of ROS. This endoplasmic reticulum stress led to a subsequent decrease of cytochrome P450 1A activity, an important xenobiotic-metabolizing enzyme (Christen and Fent, 2012). Co-exposure of these NPs with other pollutants such as the carcinogenic polycyclic aromatic hydrocarbons may thus lead to enhanced toxicity due to altered detoxification mechanisms.

7.3.5 Endocytic pathways

Uptake mechanisms could also influence the toxicity of nanomaterials. Depending on the size, surface coating and their chemical nature, nanomaterials could enter cells by phagocytosis, macropinocytosis, clathrin-dependent endocytosis, caveolin dependent endocytosis or crossing of membranes by diffusion. The size dependent toxicity of TiO_2 NPs has thus been suggested to be due to the use of different endocytic pathways (Scherbart et al., 2011). The cellular localization of nanomaterials may be particularly important in determining their toxicity. For instance, iron oxide NPs may exert catalase-like activities at cellular pH but in acidic environments like lysosomes they have peroxidase-like activity, resulting in the catalysis of H_2O_2 into hydroxyl radicals (Chen et al., 2012a). Furthermore, lysosomes present acidic pH conditions which could favor nanomaterial dissolution and even low-solubility NPs such as SiO_2 have been shown to dissolve over time within cells (Quignard et al., 2012). This solubilization is particularly important for nanomaterials releasing toxic ions such as Zn^{2+} or Ag^{2+} or transition metal ions, able to generate ROS through Fenton reactions. The effect of ZnO NPs has been shown to be greater than Zn^{2+} ions (Shen et al., 2013) which could be explained by the easy uptake of nanomaterials through classic endocytic mechanisms which facilitate the entry of toxic ions by the so-called “Trojan horse effect”, circumventing the cellular protection mechanisms. Nanomaterials present within the lysosmes could also lead to their destabilization and release of lysosomal enzymes and cellular acidification. Lysosomal rupture by nanomaterials could for instance induce the release of the pro-apoptotic protein cathepsin (*see* 7.6) which could also activate the inflammasome. The uptake mechanism could thus have great importance for the toxicity of nanomaterials as it determines the capacity to enter the cells and the intracellular fate of the nanomaterial. As mentioned earlier, the use of classic phagocytic pathways by macrophages to capture CNT could also lead to frustrated phagocytosis due to the high-aspect ratio of these nanomaterials (Murphy et al., 2012).

7.3.6 Membrane dependent signaling pathways

Nanomaterials could increase lipid mediators such as leukotrienes and prostaglandins, which have pro- and anti-inflammatory effects as shown for CB NPs in allergic animals (Beck-Speier et al., 2012). Nanomaterials could also induce membrane receptor signaling such as

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shown for TiO_2 NPs which interact with toll-like receptors (Mano et al., 2013). Furthermore, CB NPs activate the epidermal growth factor receptor (EGFR), due to ceramide accumulation in lipid rafts (Peuschel et al., 2012). This lipid raft signaling induced by CB NPs has been shown to be dependent on oxidative stress induction. Interestingly, the compatible solute ectoine prevented, ceramide-EGFR signaling and the subsequent inflammation *in vivo* (Peuschel et al., 2012). Lipid raft formation is also necessary for the induction of IL-1 beta secretion by TiO_2 (Morishige et al., 2010) and CB NPs can induce integrin mediated signaling (Weissenberg et al., 2010). Ag, Au and iron oxide NPs on the other hand could disrupt EGFR signaling through mechanisms dependent on the NP type (Comfort et al., 2011). Nanomaterials could also alter receptor-ligand interactions. For instance, Pan et al. have shown recently that Au NPs can inhibit the interaction of VEGF165 (a splice variant of vascular endothelial growth factor A) with its receptor (VEGFR2), leading to decreased Akt phosphorylation and subsequently anti-angiogenic effects (Pan et al., 2013).

7.3.7 Epigenetic regulations

MicroRNAs, small non-protein coding RNAs which regulate gene expression, have been shown to be upregulated by nanomaterials. Au NPs upregulated miR-155 leading to the down-regulation of its target gene (Ng et al., 2011) and WC-Co NPs induced miR-21 signaling (Hou et al., 2013). Citrullination of proteins was also reported for several nanomaterials (Mohamed et al., 2012). This post-translational modification converts arginine residues into citrulline and is involved in gene expression modulation through histone modification.

7.4 Interaction of nanomaterials with proteins and impact on cell signaling

In addition to the mechanisms described above, “alteration” (activation or inhibition) of cell signaling pathways by nanomaterials may also rely on NP-protein interactions (Monopoli et al., 2012). It is now well known that when nanomaterials enter a physiological environment, they rapidly adsorb biomolecules (Walkey and Chan, 2012). In particular, proteins bind to the surface of nanomaterials to form a “biological coat” around the nanomaterial, which is known as the protein corona (Walczyk et al., 2010, Monopoli et al., 2012). Several studies have been conducted to understand how nanomaterials can influence the structural properties of bound proteins and how “coated-proteins” influence the physical properties of the nanomaterials (Walkey and Chan, 2012). The protein corona is increasingly recognized as playing a major role in the biological effects of nanomaterials (Tenzer et al., 2013). For instance, binding of proteins to nanomaterials may impair their functions by alteration of their structure (Walkey and Chan, 2012). More importantly, as the protein corona is what interfaces with the cell, surface-bound proteins can drive cell-specific uptake as well as activation/impairment of cell signaling events (Walkey and Chan, 2012). Recent studies with silica and polystyrene NPs confirm that a NP-corona forms rapidly and can drive NP uptake, thrombocyte activation and endothelial cell death (Tenzer et al., 2013).

As stated above, binding of proteins to nanomaterials can impair protein functions through alteration of their structures. In addition, changes in protein structure can lead to the exposure of amino acid regions that are normally buried within the folded protein. These

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newly exposed regions can interact with other macromolecules such as cell surface receptors and hence influence nanomaterial uptake, biodistribution, receptor activation and signaling. These mechanisms have been well exemplified by studies conducted by the Minchin group (Figure 7.2)(Deng et al., 2011). These authors have shown that certain negatively charged NPs (poly-acrylic acid-conjugated Au NPs) are able to bind and unfold plasma fibrinogen in a way that leads to the exposure of a cryptic sequence in the C-terminus of the α-chain of fibrinogen. This "new epitope" interacts specifically with Mac-1 receptor in human monocytic cells. Activation of the receptor induces the NF-κB signaling pathway with subsequent release of inflammatory cytokines. Interestingly, these effects were observed only with 5 nm NPs but not with 20 nm NPs. Whereas the pro-inflammatory properties of different NPs have mostly been linked with their ability to induce oxidative stress, this study indicates that other mechanisms involving NP- protein interactions may also be at play (Deng et al., 2011).

Other studies have recently underlined that certain biological effects of nanomaterials can be driven by activation/inactivation of receptor-dependent signaling that in turn regulates cellular properties such as viability, proliferation, differentiation or cell cycle. Rosso et al. (2013) have shown that plasma vitronectin bound to certain nanomaterials (maleic anhydride/alkyl vinyl ethers-based NPs) triggers activation of the vitronectin-integrin receptor. This activation leads to increased phosphorylation of ERK1/2 and FAK kinases and increased proliferation and cell cycle progression. In addition, the authors found that the NPs were internalized by cells through a direct interaction between the NPs and the vitronectin-integrin receptor (Rosso et al., 2013).

Interactions of nanomaterials in cells with intracellular proteins with subsequent biological consequences have been reported previously (Sanfins et al., 2011). Chen and von Mikecz have reported that SiO_2 NPs could enter the cell nucleus and contribute to the formation of nucleoplasmic protein aggregates similar to those found in certain neurodegenerative diseases. Such protein aggregation could be due to interaction of nanomaterials with intracellular/nuclear proteins, the nanomaterials acting as protein aggregation anchors (Chen and von Mikecz, 2005). More recently, it was suggested that alteration of cell cycle, DNA repair and inflammatory responses in human lung fibroblast cells by exposure to Ag NPs could also be due to the capability of these NPs to adsorb cytosolic proteins on their surface (Asharani et al., 2012).

Overall, these studies underline the important role that protein-nanomaterial interactions and protein corona may play in the biological outcome of nanomaterial exposure.

7.5 Nanomaterials and cell death signaling

Complex molecular mechanisms that govern the fate of cells are becoming increasingly well understood. It is now well established that the mode of cell death dictates the homeostasis and sometimes even fate of the organism. Studies have confirmed the important role of different cell death modalities in human diseases like sepsis, neurodegenerative disorders, stroke and cancer (Galluzzi et al., 2007, Duprez et al., 2009) and therapeutic strategies based on the modulation of cell death signaling are currently being tested for treatment purposes.

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Nanomaterials can induce cell death through different modalities depending upon their physico-chemical characteristics. A detailed description of how changes in physico-chemical characteristics influence the toxicity of nanomaterials is discussed later in this chapter. Various nanomaterials can induce either programmed cell death pathways (apoptosis, autophagy, pyroptosis, and programmed necrosis) or non-programmed death pathways (accidental necrosis etc).

7.5.1 Different modes of cell death and their physiological and pathological significance

First definition of different forms of cell death came from Schweichel and Merker in 1973 (Schweichel and Merker, 1973). They classified cell death into type 1 cell death (heterophagy), type II cell death (autophagy), and type III cell death (not associated with any type of digestion), definitions that correspond to the more modern concepts of apoptosis, autophagy, and necrosis, respectively. Since the first description of cell death mechanisms in 1960s, the most commonly used criteria to define different forms of cell death are based on morphological characteristics (Kerr, 1965, Schweichel and Merker, 1973). However, the presence of particular morphology is not sufficient to establish a cause-effect link between given process and cell death (Galluzzi et al., 2012). The Nomenclature Committee on Cell Death (NCCD) has published recommendations on defining various sub-routines of cell death and has encouraged the use of specific measureable biochemical events (Kroemer and Jaattela, 2005, Kroemer et al., 2009, Galluzzi et al., 2012). Cell death can be defined either based on morphological criteria (apoptosis, necrosis, autophagy), enzymatic criteria (with or without involvement of nucleases and proteases), functional aspects (programmed vs. accidental, pathological vs. physiological) or immunological characteristics (immunogenic vs. non-immunogenic) (Galluzzi et al., 2007).

Although there are more than 10 cell death modalities that can occur under physiological or pathological conditions, only few have been reported to occur after nanomaterial exposures. A brief description of cell death mechanisms induced by various nanomaterials with their physiological and pathological significance is given below.

Apoptosis was originally defined by Ker as a type of the cell death that occurs with rounding up of cell, reduction in cell volume (pyknosis), condensation of chromatin, fragmentation of nucleus (karyorhexis), plasma membrane blebbing, and maintainance of the plasma membrane untill later stages of the process (Kerr et al., 1972). Apoptosis can be broadly classified as extrinsic apoptosis (due to extracellular signals transmitted through transmembrane receptors) or intrinsic apoptosis (due to a plethora of intracellular events/damages). Intrinsic apoptosis is further divided into caspase dependent and caspase independent intrinsic apoptosis. Apoptosis refers to a controlled/programmed process of removal of individual cells inside the body without destruction or damage to the organism. During embryogenesis apoptosis serves an important function in organ and tissue development/remodeling. Apoptosis of host cells occurs as a defense strategy in bacterial and viral infections to control the spread of infection. Moreover, apoptosis plays an important role in maintaining homeostasis and terminating an immune response (by removal of activated immune cells) (Duprez et al., 2009). Various pathological situations occur in case of deregulated apoptosis e.g. insufficient apoptosis occurs in cancers and autoimmune

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diseases while excessive apoptosis contributes to damage caused by neurodegenerative diseases, sepsis, stroke and myocardial infarction.

TiO_2 NPs induced apoptosis has been reported in a variety of cellular systems either through extrinsic or intrinsic pathways. Hussain et al. reported the comparative toxicological mechanisms induced by titanium dioxide and CB NPs in human bronchial epithelial cells (Hussain et al., 2010). Authors dissected signaling events leading to similar outcome (apoptosis) and found a significant contribution of chemical composition in downstream signaling events. TiO_2 NPs were shown to induce cell death through ROS dependent extrinsic and intrinsic pathway in human lung fibroblast and breast epithelial cells (Yoo et al., 2012). Moreover, TiO_2 NPs induced cell death in BECs was shown to involve caspase 8/Bid pathway (Kang et al., 2009, Shi et al., 2010). Lipid peroxidation, p53 mediated DNA damage and caspase activation were observed after TiO_2 nanotubes exposure in neuronal stem cells at ≥150μg/mL doses (Kang et al., 2009, Park et al., 2008). This discrepancy in the observed mechanisms is most likely due to use of TiO_2 NPs of different crystal structure, differences in cell types and dose/duration of exposures. Other metal oxides like CeO_2, ZnO and NiO were also shown to induce apoptosis. Nano CeO_2 was shown to induce ROS and caspase independent apoptosis through the release of AIF in human peripheral blood monocytes at relatively realistic exposure concentrations (10μg/mL) (Hussain et al., 2012). ZnO nanorods induced apoptosis in A549 cells through a p53 survivin, mitochondrial pathway through oxidative stress (Ahamed et al., 2011). Nickel oxide NPs induce classic intrinsic apoptosis in human airway epithelial and breast cancer cells in contrast to Ni NPs which induce apoptosis through an extrinsic pathway (Siddiqui et al., 2012, Zhao et al., 2009). Various metal NPs have also shown abilities to induce apoptosis including Ag, Au, and Cu. Ag NPs induce ER stress dependent apoptosis (Zhang et al., 2012) and Tsai et al. reported that Au NPs induce apoptosis in K562 through ER stress (Tsai et al., 2011). Nano copper induces both extrinsic and intrinsic apoptosis in mice kidney tissue (Sarkar et al., 2011).

Many biodegradable nanomaterials also induce apoptosis. Polyamidoamine (PMAM) dendrimer exposure leads to lysosomal damage induced apoptosis in KB cells (Thomas et al., 2009). Moreover, PMAM dendrimers induce mitochondrial toxicity in human lung cells (Lee et al., 2009) and polystyrene NPs (60nm) exhibited charge dependent toxicity and lysosomal damage in RAW264.7 cells (Xia et al., 2008b).

Many carbon based nanomaterials also induce apoptosis. CB NPs were shown to induce apoptosis through the mitochondrial pathway depending on ROS production and caspase activation (Hussain et al., 2010). Both single walled carbon nanotubes (SWCNT) and multi-walled carbon nanotubes (MWCNTs) have the ability to induce cell death. Multiple studies have shown apoptosis inducing properties of SWCNTs both *in vitro* and *in vivo* (Shvedova et al., 2010, Wang et al., 2011, Tyurina et al., 2011). Recently, Fujita et al. reported that the toxicity of SWCNTs depends on size and length of the bundles of dispersed nanotubes resulting in differential responses even for the same bulk SWCNTs. They reported that SWCNT were not toxic to A549 cells and the residual metals may not be a definitive parameter for intracellular ROS generation (Fujita et al., 2013).

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Autophagy is an evolutionary conserved catabolic process, which is a slow and spatially restricted phenomenon. It involves sequestration of parts of cytoplasm in double membrane bound vesicles and digestion of these components by lysosomal hydrolases (Kroemer and Jaattela, 2005). Autophagic cell death refers to cell death occurring with autophagy and should not be confused with cell death through autophagy (Galluzzi et al., 2007). Autophagy may either contribute to cell death or may constitute to a cellular defense against nutrients or growth factor deprivation induced stress. Autophagy is characterized by lack of chromatin condensation and huge increase in the number of double membrane vacuoles (autophagic vacuoles) in the cytoplasm. It has been shown that autophagy not only plays an important physiological role in removal/recycling of damaged cellular organelles but also helps in defense against bacterial infections and in immune response against viruses (through antigen presentation) (Nakagawa et al., 2004, Paludan et al., 2005). The role of autophagy in cell death during ischemia/reperfusion injury and in HIV-1 induced CD4+ T lymphocyte cell death has also been demonstrated.

An excellent review about autophagy induction and lysosomal impairment by nanomaterials has been recently published (Stern et al., 2012). Various mechanisms are described in this manuscript through which nanomaterials interact with the autophagy pathways and include overloading of lysosomes, inhibition of lysosomal enzymes and disruption of cytoskeleton-mediated vesicular trafficking leading to a state of autophagy dysfunction. Bare-Fe NPs induce oxidative stress and activation of ERK pathway in association with autophagic vesicle accumulation in RAW264.7 cells (Park et al., 2013). Au, iron core-Au shell NPs and iron oxide NPs show cell death associated with autophagic vesicle accumulation (Li et al., 2010, Wu et al., 2011, Khan et al., 2012). Au NPs induce size dependent lysosomal impairment and autophagic vesicle formation (Ma et al., 2011). ZnO NPs induce apoptosis and necrosis in RAW264.7 macrophages through p47phox- and Nrf2- independent manner (Wilhelmi et al., 2013). CeO_2 NPs were shown to induce autophagy in human peripheral blood monocytes that contributed to the cytotoxic effects of nano ceria (Hussain et al., 2012). A highly purified form of MWCNTs (vapor-grown carbon fiber, HTT2800) was shown to induce LC3b expression in human bronchial epithelial cells (Tsukahara et al., 2013). Fullerenes were shown to induce concentration dependent toxicity and induced necrosis at high doses while autophagy was observed at low doses (Harhaji et al., 2007). Quantum dots (QD) induce autophagy in human mesenchymal stem cells in a size dependent manner (Seleverstov et al., 2006) and CdSe QDs were shown to induce autophagy in porcine kidney cells (Stern et al., 2008, Johnson-Lyles et al., 2010). PMAM dendrimers promote acute lung injury *in vivo* and autophagic cell death through Akt-TSC2-mTOR signaling (Liu et al., 2011). The induction of autophagy by nanomaterials may also be exploited for therapeutic purposes (review of Stern et al. 2012).

Pyroptosis is a caspase-1 dependent cell death leading to release of inflammatory mediators (IL-1β and IL-18). It can be effectively blocked by the use of specific inhibitors of caspase-1or its genetic knockout (Galluzzi et al., 2012). Pyroptosis protects from infection and induces pathological inflammation (Bergsbaken et al., 2009). Pyroptosis occurs in various pathophysiological situations like stroke, bacterial and viral infections (Fink and Cookson, 2005, Kepp et al., 2010). This phenomenon can affect the homeostasis in multiple

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ways (local tissue damage and released inflammatory mediators cause the influx of inflammatory/immune cells) and contributes to the pathogenesis of different diseases like asthma and COPD. CeO_2 nanorods and wires, at lengths ≥200 nm and aspect ratio ≥22, have been shown to induce progressive pro-inflammatory effects (IL-1β release) and cytotoxicity of THP-1 cells (Ji et al., 2012). CB NPs can induce inflammasome dependent pyroptosis in RAW274.6 cells. Cell death induction and inflammatory responses can be effectively modulated by using caspase-1 inhibitor or pyroptosis inhibitor (Reisetter et al., 2011). MWCNT exposure leads to significant inflammasome dependent pyroptosis in primary human bronchial epithelial cells which in turn activates a pro-fibrotic response in human fibroblasts (Hussain et al. Submitted).

Necrosis was originally defined as cell death, which does not present characteristics of apoptosis and autophagy. For decades it was assumed that necrosis is an accidental form of cell death, however recently many researchers have confirmed the existence of programmed necrosis (Hitomi et al., 2008). Nanomaterials that induce necrosis include Au, Ag, TiO_2, and fullerenes (Pan et al., 2009, Kim et al., 2012, Harhaji et al., 2007). Unfortunately, no specific biochemical test exists for the confirmation of necrosis per se but another specific type of regulated necrosis (necroptosis) can be identified by the ability of RIP-1 kinase to inhibit it (Vandenabeele et al., 2010). Nano graphene oxide was shown to induce Toll-like Receptor 4 (TLR4) dependent necrosis in macrophages (Qu et al., 2013a). Go induced macrophage cell death was partially attributed to RIP1-RIP3 complex-mediated programmed necrosis downstream of TNF-α induction.

7.5.2 Cell type specificity and sensitivity

Cell type specificity/sensitivity has been observed in cell death mechanism induction by many different nanomaterials. Polystyrene NPs were shown to be toxic in RAW macrophages and BAES-2B epithelial cells but fail to induce a toxic response in endothelial cells at comparable doses (Xia et al., 2008a). Similarly, palladium NPs were shown to be toxic in bronchial epithelial cells but no toxicity was observed in case of A549 cells (Wilkinson et al., 2011). Au NPs demonstrate higher toxicity in K562 leukemia cells but are non-toxic in mononuclear cells (Tsai et al., 2011). Alili et al. (2011) demonstrated that ceria NPs preferentially killed SCL-1 squamous carcinoma cells through ROS production and oxidation of proteins at the same concentrations (150 μM), which were not toxic to dermal fibroblasts (Alili et al., 2011). Ceria NPs, which induce apoptosis and autophagy in human peripheral blood monocytes, are non-toxic to primary human bronchial epithelial cells at comparable doses (Hussain S, unpublished data). Moreover, the toxicity of nano ceriaon human monocytes and monocyte derived macrophages is cell differentiation stage dependent. This resistance appears to be related to the higher capacity of macrophages to resist mitochondrial damage (Hussain S, unpublished data). The differential sensitivity of various cell types could also be attributed to the differences in uptake mechanisms and differences in the ability to handle with oxidative insult (total anti-oxidant capacity).

7.5.3 Shared mechanisms and identification of key triggers

A review of the literature indicates that the lysosomal compartment plays an important role in the toxicity of various groups of nanomaterials. This range from the pH dependent

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dissolution of soluble metal oxides (e.g ZnO NPs) and physical damage to the lysosomal membrane by the nanomaterials (e.g CNTs) to the ROS dependent damage to the lysosomal membrane (e.g TiO_2 NPs). These events lead to the release of lysosomal proteases resulting in diverse outcomes, ranging from inflammasome activation and pyroptosis to caspase activation and necrosis.

Another main pathway involves mitochondrial damage resulting in either caspase-dependent (through activation of caspase 3/7 and caspase 9) or caspase-independent apoptosis (through the release of apoptosis inducing factor, AIF). Mitochondrial damage can take place either due to ROS generated on the surface of nanomaterials or due to physical damage to the mitochondrial membrane. The disturbed oxidant balance can occur either through the increased ROS production or through a defective antioxidant defense in response to persistent nanomaterial exposure (as postulated in case of ceria NPs).

Taken together, these results indicate that nanomaterial induced cell death signaling occurs through the classical cell death pathways and any “nano specific” cell death modality has not yet been discovered.

7.5.4 Interplay between various cell death modalities

Various nanomaterials can induce distinct cell death pathways in the same cell type depending upon their various physicochemical factors including chemical composition, size etc. (Hussain et al., 2010, Pan et al., 2009). Some nanomaterials have been shown to induce multiple cell death modalities after single exposure (Hussain et al., 2012, Hussain and Garantziotis, 2013). This question of interplay between different pathways is very complex as one mechanism might be the result of other mechanisms or a defense strategy of the body. One particular pathway that has both pro-survival and pro-death roles is autophagy. It has always been intriguing to establish the exact role of autophagy in a particular exposure. The most important question in case of autophagy is to clarify whether cells are dying due to autophagy, due to some process that induces autophagy in parallel, or is autophagy as a pro-survival mechanism. Moreover, studies have shown that cell death with autophagic vesicle detection may be completely independent from autophagic vesicle accumulation in the cells (Fimia et al., 2013). For more details the reader is referred to an excellent publication that reviewed this topic (Kroemer and Levine, 2008). Various biochemical and genetic approaches can help dissect this issue in detail.

7.6 Role of physico-chemical characteristics in nanomaterial toxicity

Biological responses to nanomaterial exposure are determined by a large diversity of factors linked to the various physico-chemical characteristics of the nanomaterials: size, surface area (taking into account the porosity and roughness of the particle), shape, bulk chemical composition (including the crystal structure), surface chemistry (including lipophilicity as well as surface charge or coatings) and surface reactivity which is linked to the two preceeding factors (surface area and surface chemistry). Various experimental studies have demonstrated the significance of these characteristics as determinants of nanomaterial biological activity/toxicity (Hussain et al., 2009, Hussain et al., 2010, Napierska et al., 2009, Pan et al., 2007, Stoeger et al., 2006). Some excellent reviews and expert opinion about this

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can be found elsewhere (Rivera Gil et al., 2010, Oberdorster et al., 2005, Oberdorster, 2010, Bouwmeester et al., 2011).

7.6.1 Size

It has been postulated that most nanomaterials have a critical size of 30 nm below which they show their typical nano characteristics as the number of atoms on the surface exponentially increases below this cutoff (Auffan et al., 2009). Studies have confirmed the higher toxic potentials of NPs compared to micrometer sized particles or larger size NPs (Carlson et al., 2008, Oberdorster et al., 2000, Napierska et al., 2009, Park et al., 2011). However, some studies demonstrate that an actual increase in size in the nanometer range is associated with higher toxicity. This stands particularly correct while studying the hemolytic potentials of nanomaterials (e.g silica (both amorphous (2–335nm) and mesoporous (100–600 nm)) (Rabolli et al., 2010, Zhao et al., 2011). It was postulated that larger particles attach to the larger surface of the red blood cells leading to membrane damage and deformity. Coradeghini et al. 2013 demonstrated size dependent toxicity on mouse fibroblasts and degradation of clathrin heavy chain after exposure to Au NPs (Coradeghini et al., 2013). Size is also a determining factor for the mode of entry into the cells as it has been shown that smallest NPs enter the cells via caveola, clathrin coated pits or lipid raft mediated uptake (Geiser et al., 2005, Nel et al., 2009, Oberdorster, 2010). Similarly, QDs show size dependence in co-localization with different organelles (Nabiev et al., 2007). Size not only determines the uptake of nanomaterials but also the interaction with proteins and the translocation potential from the site of deposition. Indeed it has been shown that Au NPs show size dependent translocation potentials (1.4 vs 18 nm) (Semmler-Behnke et al., 2008).

7.6.2 Surface area and porosity

The surface area of nanomaterials is not only dependent on the size but also on their porosity. It was shown that mesoporous Si NPs show higher biocompatibility and less hemolytic potentials as compared to the particles of same size but different porosity (Lu et al., 2007). For the equal mass of particles with same chemical composition and crystalline structure, a greater toxicity was found for NPs than for larger particles. This led to the conclusion that inflammatory effects of nanomaterials depend upon the surface area (Buzea et al., 2007). A surface area dependent inflammatory response after inhalation or instillation has been shown for various nanomaterials including CB, TiO_2 or Ni (Donaldson et al., 2002, Oberdorster et al., 2000, Oberdorster et al., 1994). It has been found that 21 nm TiO_2 was 43 fold more potent to induce pulmonary inflammation than 250 nm particles (Oberdorster et al., 1994). It was demonstrated that titanium dioxide NPs instilled at same surface dose but different mass exert similar toxic responses fitting the same response curve. Hussain et al. 2009 showed a direct correlation between the particle surface area and potentials to induce pro-inflammatory and oxidative responses in bronchial epithelial cells exposed to CB or TiO_2 NPs. Some studies contradicted the significance of surface area dose metric (Warheit et al., 2006, Wittmaack, 2007). Wittmaack *et al*. (2007) analyzed the data already published by Stoeger et al (2006) and Oberdorster et al (2005) and suggested that particle number seemed to be the best dose metric rather than surface area. Furthermore, Warheit and colleagues concluded that smaller and larger NPs respond in a similar manner in cytotoxicity testing (Warheit et al., 2006).

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7.6.3 Crystalline structure

Nanomaterials of same chemical composition can have different crystalline structures, which can potentially influence the toxicity of the material. The most widely used example to elaborate such effects is TiO_2, which has many crystalline forms out of which anatase, rutile and brookite are most actively studied. Sayes *et al*. (2006) demonstrated that the anatase form of titanium dioxide is 100 times more toxic than the same mass of rutile form and that the ROS production after UV illumination follows a similar trend as the biological activity (Sayes et al., 2006b). However, another literature report contradicted these results and demonstrated that the rutile TiO_2 induces oxidative DNA damage in the absence of light but anatase NPs of same size did not (Gurr et al., 2005). Jiang *et al* (2008) did a comprehensive study on model TiO_2 NPs. Different sizes (3–200 nm) of anatase, rutile or anatase/rutile mixtures of different ratios and amorphous TiO_2 were compared with regard to their ability to produce ROS in a cell-free phosphate buffer assay. Based on their experimentation they ranked NPs from highest to lowest reactivity as amorphous > anatase > anatase/rutile mixture > rutile (Jiang et al., 2008). Furthermore, they normalized the ROS-producing abilities to NP surface area and found striking size dependence. They observed that NPs between 3–10 nm have about the same ROS production abilities per unit surface area followed by a steep increase between 10 and 30 nm and a constant but still higher ROS production capacity per unit surface area between 50 and 200 nm. The authors suggested that this finding is due to higher number of defects per unit surface area in larger anatase NPs as compared to smaller ones.

7.6.4 Chemical composition

Particle chemistry is critical for the toxic potential of nanomaterials. Although it was suggested that size is more important than chemical composition in the toxicity of nanomaterials, the extrapolation of the results showing similar extent of inflammation from different chemical compositions is not possible (Risom et al., 2005). Hussain *et al*. 2010 showed that chemical composition dictates the nature of intracellular cell death signaling as CB and TiO_2 NPs of comparable sizes induce distinct cell death pathways. Wang *et al* showed that the toxicity of QD depend upon their composition as CdSe QD are more toxic than CdTe whereas ZnS-AgInS2 QD were much less toxic (Wang et al., 2010). This cytotoxicity was attributed to the leakage of highly toxic cadmium ions. Indeed, the solubility of the nanomaterials is a critical feature for their toxicity. The biological effects could either be increased by insolubility leading to biopersistance of the material or in contrast by dissolution as also observed for ZnO and CuO NPs which toxicity could be attributed to leaching of Zn or Cu ions (Mortimer et al., 2010). However, particle associated toxicity was also observed in other studies using ZnO NPs (Moos et al., 2010). It is important to note that dissolution kinetic is size-dependent and therefore an important factor to be considered for nanomaterial toxicity though several other physico-chemical characteristics influence dissolution kinetics: particle surface characteristics such as roughness or curvature influence the kinetics of dissolution and adsorbed molecules could either slower solubility or serve to catalyse dissolution. The aggregation state of the particles needs also to be considered as a hindering factor of solubility (Borm et al., 2006). It is interesting to note that acidity could favor dissolution and thus preferential uptake of

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nanomaterials into lysosomes or the less acidic caveolar compartment could thus influence the fate of the particles as shown for ZnO and CeO NPs (Xia et al., 2008a).

7.6.5 Aggregation and Concentration

The concentration dependency of nanomaterial-induced effects is not clear enough. One important factor, which is usually ignored while interpreting these results is the state of aggregation of the nanomaterials, which is important in determining the extent of internalization and clearance of the nanomaterials. Indeed, aggregation is dependent on the concentration of the test substance and higher nanomaterial concentrations have been shown to promote aggregation (Churg et al., 1998)(Gurr et al., 2005, Nel et al., 2009). This results in reduced toxicity as compared to that observed at lower concentrations (Takenaka et al., 2001). It is postulated that aggregation depend upon surface charge, material type, size etc. (Buzea et al., 2007). Most of the times the observed aggregates are larger than the threshold limit for the biological responses and thus result in no toxicity.

The agglomeration state not only increases the size of the nanomaterial influencing its lung penetration, deposition and cellular uptake but also the solubility of the material (Borm et al., 2006). This factor also determines the extent of translocation across cellular barriers and biokinetics inside the body. The physico-chemical surface properties of the material such as charge and hydrophobicity are the main determinants for the degree of aggregation but characteristics of the suspending media (pH, viscosity, ionic strength etc.) also play a role. Thus coatings of derivative groups as well as dispersants may allow the stabilization of nanomaterials by preventing the formation of aggregates, which has an effect on toxicity. For instance dispersion of SWCNT by the use of surfactant (Wick et al., 2007) or by adding functional groups (Sayes et al., 2006a) reduces effectively the cytotoxicity.

7.6.6 Aspect ratio

A direct relationship exists between aspect ratio and toxicity. CNTs are classical examples of engineered long aspect ratio nanomaterials. These materials have many similarities with asbestos but untill now there is no agreement on their toxic potential. These materials exist in a multitude of morphologies, sizes and surface/end functionalizations which make their risk evaluation increasingly difficult. Typically CNTs have diameters between 0.4 nm –100 nm and lengths from a few nanometers to several centimeters. It has been shown that when administered in similar doses, CNTs have higher toxic potentials as compared to spherical particles (CB, silica). In this study authors demonstrated that SWCNTs induce granuloma, alveolar wall thickening, acute inflammation and progressive fibrosis and these effects were attributed to their physicochemical properties and fibrous nature (Shvedova et al., 2007). High aspect ratio nanomaterials (HARN) can indeed induce frustrated phagocytosis leading to inflammation (Murphy et al., 2012). CeO_2 nano-rods and wires, at lengths ≥200 nm and aspect ratio ≥22, have been shown to induce progressive pro-inflammatory effects (IL-1β release) and cytotoxicity of THP-1 cells (Ji et al., 2012). These findings suggested that both, length and diameter components of aspect ratio, should be considered while addressing the toxic effects of high aspect ratio nanomaterials.

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7.6.7 Surface-coating/modifications

As described earlier the surface of nanomaterials is an important determinant for their toxic potential. A thorough understanding of nanomaterial surface composition helps in defining the interactions of nanomaterials with biological systems. Such surface modifications can be either deliberate or unintended. Unintended surface modifications arise from the interaction of nanomaterials with their environment (air/liquid) resulting in the attachment of environmental components. Presence of oxygen, ozone, oxygen free radicals and metals on the surface of nanomaterials can lead to enhanced potentials to produces ROS. Absorption of LPS and bacterial endotoxins to nanomaterials is a common problem as these are ubiquitous in nature and can play an important role in the biological responses to nanomaterials (Vallhov et al., 2006). Surface modifications of engineered nanomaterials during the production process lead to surface functionalization and influence their toxicity. Acidic treatment of MWCNT leads to more carboxyl, carbonyl and hydroxyl groups and thus increases the cytotoxicity (Magrez et al., 2006) but on the other hand functionalization that increases the water-solubility of SWCNT leads to decreased cytotoxicity (Sayes et al., 2006a). Functionalization dependent decrease in toxicity of MWCNT was also observed (Li et al., 2013). It has been shown that surface oleic acid modifications influence iron oxide and nickel ferrite particle cytotoxicity (Guadagnini et al., 2013). Recently it has been shown that uptake and toxicity of sub-10nm cerium oxide depend upon the surface coating with citrate coated NPs having higher potentials of internalization than polymer-coated NPs (Safi et al., 2010). Moreover, it has been shown that toxicity of QD can be mitigated by appropriate functionalization (Derfus et al., 2007). Agglomeration of the NPs occurring concomitant with ageing may be a contributing factor to this decrease in toxicity. Moreover, structural defects on the surface of MWCNT are mainly responsible for the pulmonary toxicity observed *in vivo* and *in vitro*. Indeed, annealing structural defects and elimination of metal contaminants by heating reduces the lung responses after intratracheal instillation but further grinding of the material restored their toxic potential (Muller et al., 2009).

7.6.8 Interplay

Beside these inherent properties of the nanomaterials the interaction between particles or molecules will also determine their toxicity. Interactions between particles of different composition could have unexpected biological consequences as seen for cobalt tungsten carbide particles known to induce hard metal lung disease. It is the contact between the particles which causes the release of ROS involved in the pathogenic response as pure cobalt or carbide particles are inert and soluble cobalt salts in contact with carbide particles have no effect (Lison et al., 1995). Recently the same effect has been observed for co-exposure to CB and Fe_2O_3 NPs leading to oxidative effects whereas exposure to either particle type alone has no effect (Guo et al., 2009). This synergistic effect is probably due to intracellular redox reactions between CB and Fe^{3+} solubilized within the lysosomal compartment leading to the generation of Fe^{2+}.

Conclusions

The diversity and complexity of the factors involved makes nanotoxicology a very challenging field. Several parameters could influence the final biological response to

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nanomaterials and it is thus difficult to predict the human health hazard after exposure. A thorough physicochemical characterization of the tested nanomaterial prior to *in vitro* or *in vivo* biological evaluation is needed to allow the comparison of data and to draw general or specific conclusions on toxicity of nanomaterials for public health risk assessments. Nanomaterials have the ability to induce either oxidative stress dependent or independent mechanisms and acellular ROS production potentials may not accurately predict the biological activity of nanomaterials. The complex interactions of nanomaterials with proteins could also impact on signaling pathways. Nanomaterials have shown the ability to induce classical cell death signaling pathways in ROS dependent or independent manners and this ability can also be correlated to different physico-chemical characteristics of nanomaterials. Taken together it becomes evident that nanomaterials are capable of either inducing complex cellular signaling mechanisms or modifying existing signaling pathways resulting in adverse/unanticipated outcomes. It is therefore essential that a mechanistic approach with detailed elaboration of cellular signaling events is adopted for the safety evaluation of nanomaterials as well as for the development of safer and consumer friendly nanotechnology.

Acknowledgments

Funding

This work was supported (in part) from the funding by the intramural program of the National institute of Environmental Health Sciences (NIEHS)/National Institute of Health (NIH) USA and the European FP7 SIINN ERA-NET project NanOxiMet.

Abbreviations

AP-1	Activator protein 1
CB	Carbon black
CNT	Carbon nanotubes
LPS	Lipopolysacchrides
MWCNT	Multi-walled carbon nanotubes
NP	Nanoparticle
NADPH	Nicotinamide adenine dinucleotide phosphate
NF-κB	Nuclear factor-kappa B
Nrf-2	Nuclear factor (erythroid-derived-2)-related factor 2
QD	Quantum dots
ROS	Reactive oxygen species
SWCNT	Single walled carbon nanotubes
UPR	Unfolded protein response

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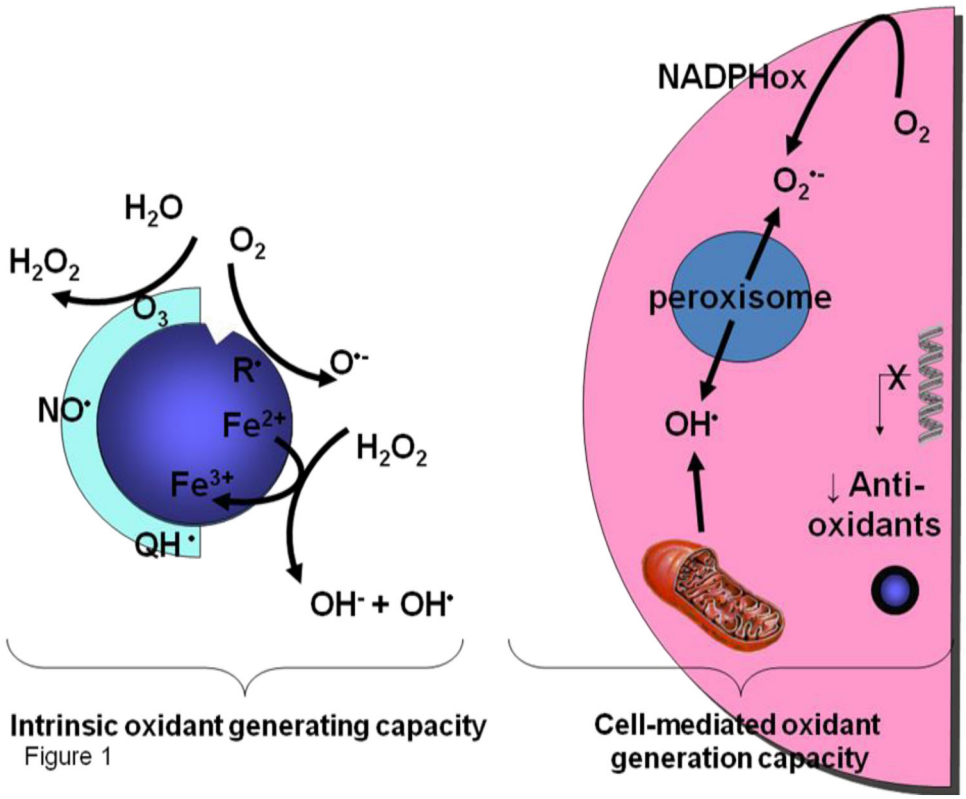

Figure 7.1.

Overview of ROS production mechanisms by nanoparticles. Nanoparticles can induce ROS production either 1) through their intrinsic ability to catalyze oxidation reduction reactions through their surfaces or 2) through interaction with cellular components and normal ROS production mechanisms (e.g mitochondria and NADPH oxidase system) or by decreasing the cellular anti-oxidant defense mechanisms (e.g. savaging/inactivation or decreased production of anti-oxidants).

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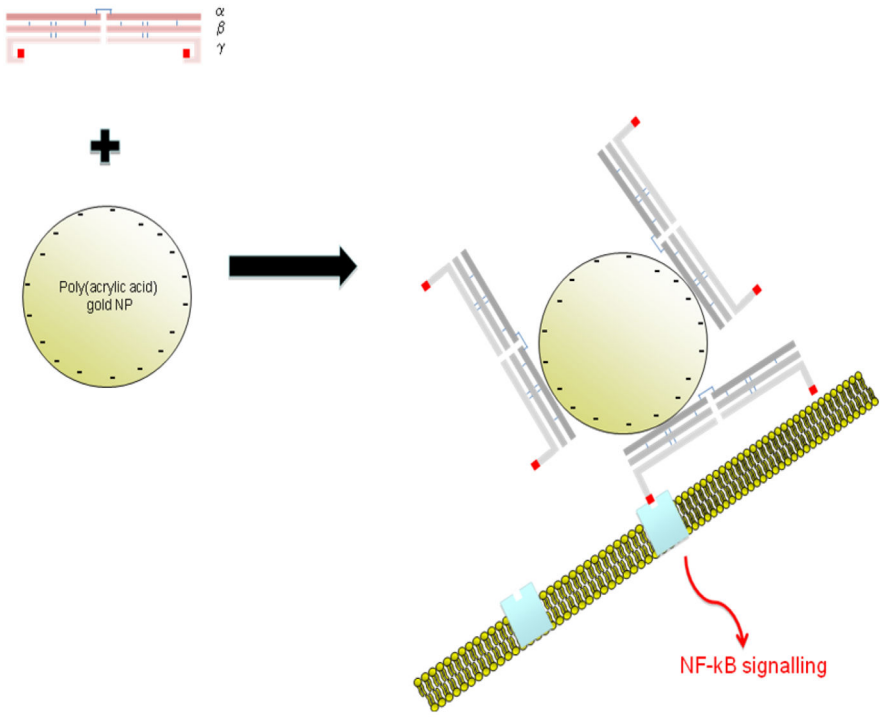

Figure 7.2.

Plasma fibrinogen is able to interact with negatively charged poly(acrylic acid) gold NP. With 5 nm NPs the binding of fibrinogen induces unfolding which exposes an amino acid sequences of the g chain. This new epitope interacts with MAC-1 receptor of monocytes, activates the NF-κB pathway which leads to pro-inflammatory cytokine release (modified from Deng et al., 2011)