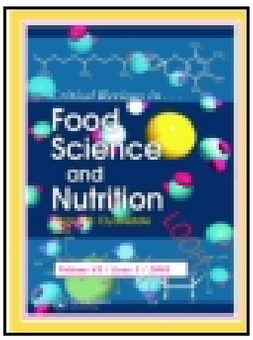

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Aditi Jain, Shivendu Ranjan, Nandita Dasgupta & Chidambaram Ramalingam

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Nanomaterials in Food and Agriculture: an overview on their safety concerns and regulatory issues

Aditi Jain[1,*], Shivendu Ranjan[2,3,4,$], Nandita Dasgupta[2], Chidambaram Ramalingam[2]

[1]Department of Biotechnology, Jaypee Institute of Information Technology, A-10, Sector-62, NOIDA, 201307, Uttar Pradesh, India

[2]Nano-Food Research Group, Instrumental and Food Analysis Laboratory, Division of Industrial Biotechnology, School of Bio Sciences and Technology, VIT University, Vellore, Tamil Nadu, India

[3]Research Wing, Veer Kunwar Singh Memorial Trust, Chapra, Bihar, India

[4]Xpert Arena Technological Services Pvt. Ltd., Chapra, Bihar, India

[*]Corresponding authors: Aditi Jain, Department of Biotechnology, Jaypee Institute of Information Technology, A-10, Sector-62, NOIDA, 201307, Uttar Pradesh, India, Email: aditijain506@gmail.com, Mobile: + (91)-9818437191

[$]Corresponding authors: Shivendu Ranjan, Research Wing, Veer Kunwar Singh Memorial Trust, Chapra, Bihar, India, Email: shivenduranjan@gmail.com, Mobile: +91-9566763718

Abstract

Nanotechnology has seen exponential growth in last decade due to its unique physicochemical properties; however, the risk associated with this emerging technology has withdrawn ample attention in the past decade. Nanotoxicity is majorly contributed to the small

size and large surface area of nanomaterials, which allow easy dispersion and invasion of anatomical barriers in human body. Unique physio-chemical properties of NPs make the investigation of their toxic consequences intricate and challenging. This makes it important to have an in-depth knowledge of different mechanisms involved in nanomaterials's action as well as toxicity. Nano-toxicity has various effects on human health and diseases as they can easily enter into the humans *via* different routes, mainly respiratory, dermal and gastrointestinalroutes. This also limits the use of nanomaterials as therapeutic and diagnostic tools. This review focuses on the nanomaterial-cell interactions leading to toxicological responses. Different mechanisms involved in nanoparticle-mediated toxicity with the main focus on oxidative stress, genotoxic and carcinogenic potential has also been discussed. Different methods and techniques used for the characterization of nanomaterials in food and other biological matrices have also been discussed in detail. Nano-toxicity on different organs - with the major focus on the cardiac and respiratory system -- have been discussed. Conclusively, the risk management of nanotoxicity is also summarized. This review provides a better understanding of the current scenario of the nanotoxicology, disease progression due to nanomaterials, and their use in the food industry and medical therapeutics. Briefly, the required rules, regulations and the need of policy makers has been discussed critically.

Keywords

Nano-foods; Cardiovascular risk; Respiratory risk; Regulations; Food toxicity; Nanomaterials risk management.

1. Introduction

Nothing is perfect in this world and every good thing has its downsides as well. The same concept goes with the newly emerging field in science- Nanotechnology. The recent advances and the advantages of the technologically important nano materials (NMs) have drawn a huge attention towards their toxicology and the relationship between nanoparticles (NPs) exposure with the onset of various diseases. The fact that NPs are associated with toxic side effects and hampers human health is not new. NPs present in the aerosols and air pollution have been studied decades back and it has been established that it leads to the onset of several cardiac and respiratory diseases (Dockery et al., 1993; Ferin et al., 1992; Kingsley et al., 2013; Nandita et al., 2015; Schwartz, 1994).NP toxicity is very complex and multifaceted as it depends on a variety of physicochemical and surface properties like their size, shape, charge, area and reactivity. The nano range particles (<100 nm) are more toxic than larger particles of identical chemical composition. It has also been observed that particle surface area dose predicts the better toxic and pathological responses to inhaled particles as compared to the particle mass dose (Fadeel and Garcia-Bennett, 2010).

Man-made/ engineered NPs have well-known applications in wide range of fields with the increasing demand in material science, electronic devices, biomedical research, food industry *etc*. Within the biomedicine industry, NP application has expanded to the areas of diagnostics and therapeutic purposes. The nano product demands in medicine and the pharmaceutical industry is expected to rise by over 17% each year and at a much higher rate in the food industry (Jones and Grainger, 2009). On the other hand, the promising field of nanotechnology has also

triggered the understanding of the unexpected and unanticipated effect of nanoscale materials on health and disease progression (Maynard et al., 2011). A systematic understanding of the NM interactions with biological systems at cellular, molecular and physiological levels is essential for understanding the possible unsafe responses. The toxicological response varies between molecular and the nano-sized forms in different organs. The adverse health effects of the NPs may take place from direct contact to the purposely used NMs and/or by-products associated with their applications. Exposure to NPs adversely affect mammals and other species at cellular, organ and tissue level by causing oxidative stress and inflammation. It also leads to the altered function of the autonomic nervous system that in turn results in enhanced respiratory and cardiovascular diseases. NPs can enter the blood circulation and migrate to different organs and tissues, and injure oxidative stress sensitive organs. In general, NPs mediated toxicology affects lungs functioning; alter heart rate and blood pressure, and displays respiratory symptoms, thrombosis, myocardial infarction, arrhythmia and strokes causing shorter life expectancy (Künzli and Tager, 2005).

In the present review, the underlying mechanism of NP-mediated toxicity has been summarized along with the different aspects of nanotoxicity including the signal transduction induced by engineered NPs, biological and physiological outcomes of NPs exposure, different toxic potentials of NPs, their toxicity evaluation methods and the effect on the major organ systems. The effect of NPs on cardiac and respiratory system has been discussed in detail.NP toxicity with respect to specific organ systems that have been carefully studied by many international specialists with different types of engineered NPs *in vivo* and *in vitro* models have been put together and studied. This review will also provide the much-needed information on the

available methodologies for the risk assessment of the NPs as well as the characterization of NMs in biological matrices and the problems associated with measurement and characterization of NMs. This in-depth study will lead to a better understanding of the current scenario of the nanotoxicology and disease progression and their use in the food industry and medical therapeutics. It also reflects a sense of urgency to understand the complexity of nanotoxicology and a need of continuous synchronization of risk management.

2. Nanoparticle-Cell interaction

NP-cell interactions are mainly dictated by the different surface properties of NPs. Unique intrinsic properties of NPs including high tensile strength, high conductivity, physiochemical, electrical and thermal properties makes it possible to interact with the cell. Different chemical moieties present on the NP surface also decide the NP interaction with cell and lipid bilayer (Verma and Stellacci, 2010). In prokaryotic cells, silver and zinc oxide NPs electrostatically interacts with the bacterial cell surface and causes toxicity. Such interactions also result in morphological and mitochondrial alterations as well as cytoplasmic accumulation of NPs within the cells (Sinha et al., 2011). On the other hand, eukaryotic semi-permeable plasma membrane selectively permits few important nano-sized molecules across the lipid membrane either by specific membranetransport protein channels or by endocytosis (Alberts et al., 1997; Conner and Schmid, 2003). For engineered NPs, crossing the lipid bilayer is difficult and only cationic nanoparticles can penetrate by creating pores in the cell membranes. This results in toxicity by generating an imbalance in intracellular and extracellular ions, proteins, and other macromolecules that are required to protect the integrity of a cell (Leroueil et al.,

2008).The shape and size of NPs greatly influence their cellular internalization and an optimum size of 50-nm and spherical shape has been found to be most efficient. These properties also affect the nature of receptor binding and activation of pathways inside the cells(Jiang et al., 2008). As the charged functional groups present on the NPs are responsible for active interaction with cells, their role has been explored in detail in various studies. The internalization of anionic NPs occur through their nonspecific binding and clustering on cationic sites present on the cell membrane followed by endocytosis (Shi et al., 2007). On the other hand, cationic NPs bind actively to the negatively charged groups on the cell surface and move across the plasma membrane at a much higher rate as compared to the cationic or neutral NPs(Martin et al., 2008).They can also be internalized through the clathrin-mediated pathway and upon inhibition of these endocytic pathways, compensatory endocytosis takes place for the faster internalization(Lee et al., 2008). Contrasting results were observed in the case of red blood cells where the surface charge and the material of the particles do not influence their entry into the cells. This may be contributed to the fact that red blood cells have no phagocytic receptors on their surface and hence NPs can penetrate easily. However, their exact mechanism of entering into the red blood cell is still not known properly(Rothen-Rutishauser et al., 2006).

Carbon nanotubes (CNTs) interact with cellular proteins and other biomacromolecules once they enter into cells and may damage some important functional proteins by the interaction of carboxylic groups present on the nanotubes and RNase A. This results in damaged protein secondary structure as well as reduced enzymatic activity(Zhang et al., 2009). CNTs with larger diameters and more functional groups are known to be less toxic to organisms(Zhao and Liu, 2012). Magnetic properties of NPs have also been exploited in many applications including

biomedicine where the main requirement is the targeted cellular uptake of NPs by cells. There also, NPs follow the mechanisms like endocytosis and pinocytosis (Berry, 2005). NPs acquire various intracellular responses depending on their physicochemical properties, intracellular concentrations, time of contact, and interactions with biological components. Endocytic pathways consist of pinocytosis, the formation of caveolae and clathrin, and caveolae/clathrin-dependent uptake(Dobrovolskaia and McNeil, 2007). NPs are known for crossing the blood brain barrier and reaching the central nervous system. Surface characteristics with polymeric matrix contribute to this phenomenon(Mailänder and Landfester, 2009).

The interaction of NPs with cells forms a solid-liquid interface. This interface is characterized to understand the complex NP-cell interactions as it undergoes continuous alterations because of the cellular microenvironmental factors. NP wrapping by the cell surface membrane for the cellular internalization leads to new interfaces. These interphases can be studied by different imaging techniques discussed in the following sections of the review (Nel et al., 2009).

3. Mechanism of Nanotoxicity

The increasing demand of NMs in different industrial sectors has also raised a question mark on the safety issues and their adverse reactions on human health. For the better understanding of these parameters, it is essential to understand the mechanisms of action of NPs as well as the nanotoxicity. The mechanisms primarily responsible for nanotoxicity have been recently studied intensively to overcome the NP limitations. One of the important mechanisms of nanotoxicity is the generation of reactive oxygen species (ROS) and oxidative stress (Li et al.,

2010; Manke et al., 2013). These chemically active free radical species further lead to an imbalance in downstream pathways by triggering DNA damage, altered cellular signaling and programmed cell death. Many studies have shown NMs to be genotoxic, inflammatory and can result in cellular death. NMs are well studied for their carcinogenic potential thereby affecting cells and tissues and complicate the proper working. By understanding the mechanism of nanotoxicity, safer NPs can be designed as well as the adverse effects of the NPs can be predicted beforehand. The current understanding of NP-mediated toxicity is discussed in detail in the following sections.

3.1. Nanoparticles-induced Oxidative stress

Within a cell, ROS plays a role in both protective and destructive ways in different pathways. ROS are the key signaling molecules for several pathways and they are generally produced as the by-products of mitochondrial electron transport chain apart from other intrinsic and extrinsic pathways like ROS generation via peroxisomes, inflammatory responses or external inducing agents. Their basal levels are maintained by the inbuilt cellular antioxidant machinery and the imbalance in this leads to oxidative stress. Different studies suggest that ROS generation is the most common phenomenon in NP-induced toxicity. Engineered NPs mediated oxidative stress is mainly because of the different cellular and acellular factors like NP size, surface properties, composition, metal ions and their reactivity, cellular interactions, immune response generation *etc*. The presence of electrons on NMs boundary is also a main factor for their high reactivity. These properties catalyze the ROS production. Different ROS-mediated cellular responses of NPs are depicted in Figure1. NMs results in ROS generation by direct or indirect

mechanisms being directly involved or via triggering reactions within the cellular inbuilt machinery. NM surface may own surface bound radicals that can react with oxygen in the cell and results in the generation of different forms of free radicals. Also, NPs may have transition metals on their surface that can trigger ROS generation in the cells. NPs with metal oxide particles cause cytotoxicity and genotoxicity by ROS generation and the same have been reported in different *in vitro* and *in vivo* systems. Nickel nanowires mediated oxidative stress leads to induce cell cycle arrest, altered mitochondrial membrane potential and apoptosis in HeLa cells (Hossain and Kleve, 2011). Silica NPs induce oxidative stress-mediated inflammation and endothelial dysfunction *in vitro* by stimulating the MAPK/Nrf2 pathway and nuclear factor-κB signaling in vascular endothelium, thus, suggesting the hazardous outcome of the silica-NP application on vascular homeostasis (Guo et al., 2015). Cobalt oxide NPs have shown to cause oxidative stress-mediated activation of TNF-α/caspase-8/p38-MAPK signalling in human leukemia cells leading to cellular death (Chattopadhyay and Dash, 2014). Zinc oxide NPs have also shown to induce apoptosis in colon carcinoma cells by oxidative stress leading to cytotoxicity by inflammatory responses, mitochondrial membrane alterations and IL-8 release in the cancerous cells (De Berardis et al., 2010). Such mechanism is helpful in treating cancers but otherwise, the key point of concern is that this free radical generation in normal cells is very injurious. NPs also elicit ROS within cells by disturbing the mitochondrial functioning and an imbalance in the electron transport chain leads to the increased ROS generation. NMs can also activate the inflammatory cells and results in respiratory burst thereby altering the functioning of membrane-boundantioxidant enzyme NADPH oxidases. High aspect ratio NMs generally activates macrophages because of their long, thin and persistentfibers. Redox homeostasis of the

cell is disturbed by the NPs because of the depletion of antioxidant cellular levels. NPs also interfere with the scavenging properties of the antioxidant enzymes and metalloproteins leading to oxidative stress. NP exposure alters different signaling pathways via oxidative stress and may lead to carcinogenesis, apoptosis or inflammation. CNT-induced oxidative stress results in upregulation of proinflammatory cytokines, macrophages and fibrotic cytokines (Jasmine et al., 2010; Kennedy et al., 2009). Being nanoscale in size, NPs have the tendency to accumulate within the cell or on the cellular surface thereby inducing the free radical generation cascades (Dhawan et al., 2009; Oberdörster et al., 2005).

3.2. Apoptosis

It has been well recognized that oxygen-derived species are having the main role in causing cell injury or death. ROS has been identified as critical signalling molecules in a number of pathways regulating both cell survival and cell death(Azad et al., 2009). Potential mechanisms of apoptosis were studied for zinc oxide NPs in cultured primary astrocytes and it was observed that it triggers dose- and time-dependent reduction in cell viability, increase in lactate dehydrogenase release, stimulate intracellular ROS generation, and elicit caspase-3 activation. Decrease in mitochondrial membrane potential with a simultaneous increase in the Bax/Bcl-2 ratio indicates the role of mitochondria in P-mediated apoptosis. Phosphorylation of c-Jun N-terminal kinase (JNK),extracellular signal-related kinases, and p38 MAPK specify the involvement of JNK signalling pathways in NP-induced apoptosis in primary astrocytes(Wang et al., 2014). Other mechanisms involved in NP-mediated apoptosis include the up-regulation of the transcription of various pro-inflammatory genes, including tumor necrosis factor-α and IL-1, IL-

6, and IL-8, by activating nuclear factor-kappa B signaling(Khanna et al., 2015).Zinc oxide NPs trigger p47NADPH oxidase-mediated ROS formation in macrophages and caspase-9/3-mediated apoptosis. Apoptotic cell death by zinc oxide NPs appears to be NADPH oxidase and Nrf2-independent and can also be triggered by alternative routes (Wilhelmi et al., 2013).

3.3. Genotoxic potential

As the NMs are absorbed through the gastrointestinal (GI) tract, it interacts with various types of cells, proteins, and even DNA. Due to its small size and high reactivity, the probability of their internalization into the cells and cellular organelles - macromolecules interactions (DNA, RNA, and proteins) are very high. These interactions can alter the genetic material, induce mutations, disturb the biochemical pathways and defense mechanisms. NMs are reported to induce genotoxicity either by direct interaction of NMs with the genetic material or indirect damage due to ROS generation and the release of toxic ions (Barnes et al., 2008; Kisin et al., 2007). NMs used in food are reported to induce ROS generation under *in vitro* and *in vivo* conditions (Heng et al., 2011; Jones and Grainger, 2009; Karlsson et al., 2009; Khan et al., 2012; Xie et al., 2010). Studies have shown NMs interaction with cytoplasmic/nuclear proteins, disturbance of cell cycle, oxidative stress, ROS generation or binding with amitotic spindle or its components. Interruption of antioxidant defence by NMs also induce genotoxicity (Ashutosh et al., 2015; Dhawan and Sharma, 2010; Kansara et al., 2015; Shukla et al., 2013b).

Using computational approach, it was observed that during DNA replication, CNTs can bind to sister DNA strand and gets integrated into DNA duplex thereby hindering the DNA replication process. Apart from CNTs, other NMs are also reported to show strong interaction with the DNA

and its bases in different organisms (An et al., 2010; Jin et al., 2012). An *in silico* study showed disturbance of DNA mismatch repair pathway by C60 fullerene by possible interaction with PMS2, RFC3, and PCNA proteins. A study by Baweja et al. (2011) computationally showed that C60 fullerene can interact with the ATP-binding domain of human DNA topoisomerase II alpha and could inhibit the enzyme activity (Baweja et al., 2011; Benyamini et al., 2006). Interaction studies of NMs and other proteins suggest that it binds to active site of the protein leading to their structural/conformational changes. Interaction with enzymes has shown competitive inhibition of the enzyme due to the inability of the substrate to bind. Jugan et al. (2012) have shown DNA repair activity in A549 cells was impaired by TiO_2NPs. The inactivation of the DNA repair protein activity has been attributed to the ROS generation (Jugan et al., 2012; Kansara et al., 2014).

Similarly, NMs were also investigated for the interaction of proteins involved in pathways regulating biological functionalities of many systems such as mitotic spindle apparatus, DNA replication, centrioles, transcription and repair; and associated proteins. The interaction studies are based on the data of various computational and *in vitro* studies. Signaling pathways can be activated by low concentrations of ROS. On the other hand, at higher concentration, it leads to lipid peroxidation and damages cell membrane, mitochondria, and other macromolecules. The major source of the oxygen-free radicals and major target of ROS-induced oxidative stress and damage is -- mitochondria. Different pro-apoptotic factors are released by mitochondria under stress condition due to the depolarization of the inter-membrane potential and an increased permeabilization of the outer membrane (Cadenas and Davies, 2000; Kumar et al., 2011a; Shukla et al., 2013a). Various modified DNA bases can be generated by direct attack of ROS on DNA

out of which the most abundant is 8-oxo- 7,8-dihydroguanine (8-oxoG), and play a major role in carcinogenesis as well as mutagenesis. 8-oxoG can be considered as an indicator for DNA damage because of oxidative stress after NMs exposure which has been analyzed by FPG-modified comet assay (Asare et al., 2012; Kim et al., 2011; Magdolenova et al., 2014). It can be noted thatlevels of 8-oxoguanine DNA glycosylase (OGG1) is found to be induced by ROS which ultimately affects base excision repair of 8-oxoG. It has been proved that in the liver of rats treated with C60 fullerene, there is an enhanced expression of mRNA of OGG1; though, a corresponding enhancement in its repair activity is not observed. The NM induced genotoxicity can be inhibited by pre-treatment with the free radical scavengers like *N*-acetyl- cysteine (NAC) (Guo et al., 2011; Sharma et al., 2012a). This ultimately helps to understand the mechanism of ROS-induced cellular perturbation along with apoptosis and DNA damage.

3.4. Carcinogenic potential

DNA damage and mutations are induced by NMs -- this fact has been established by several *in vitro* and *in vivo* experimentations and an association between genotoxicity and cancer is already known. Therefore, this analysisprovides very useful information in expecting the carcinogenicity of NMs, for example, ability to cause gene mutations and DNA damage of the physicochemical factors such as UV radiation, ionizing radiation, and many chemical carcinogens. The correlations of metallic, metal oxide and organic molecules with oxidative stress, and cancer have been much explored in research and reviews (Barchowsky and O'Hara, 2003; Lee et al., 2012; Pulido and Parrish, 2003; Valko et al., 2005). A Large number of degenerative changes which leads to tissue degradation because of involvement of ROS that ultimately causes

carcinogenesis, aging, and other diseases (Luo et al., 2011). Additionally, it also affects immune system which further leads to an increased microbial load and result in cell and tissue damage. Cancer causing different types of genetic changes is produced by the free radicals, among which 8-OHdG is the most studied because of its relative premutagenic potential and ease of measurement. Notably, in many tumours elevation of 8-OHdG has been reported which strongly associates such damages in the etiology of cancer. Several cell lines based studies suggest the carcinogenic potential of NMs is because of their capability to induce the level of 8-OHdG in cells.

Oxidative stress acts as on initiator of carcinogenesis and leads to inflammatory responses. NMs react with proteins and enzymes at faster rate and adsorb endogenous substances and trigger cytokine release which is responsible for mediating inflammatory responses and potentially instigate a series of toxic responses (Bergamaschi et al., 2006; Borm and Kreyling, 2004). C60 fullerene can be taken as the best example in this regard since it causes photo-induced DNA damage by interacting with NADH. It can be noted that NADH is an endogenously natural reducing agent present in cells (Wang et al., 2009; Yamakoshi et al., 2014).

4. Toxicity evaluation methods and techniques used

Various models including *in silico*, microbial system, cell culture *in vitro* and *in vivo* models can be used to assess the genotoxicity of the NMs. The Ames test has been extensively accepted to assess the genotoxicity of a variety of NMs (Kumar et al., 2011b; Maenosono et al., 2009; Sotto et al., 2009). Ames test or bacterial reverse mutation assay can be done for early screening of genotoxicity. It is used to detect mutagenesis based on the reversion of histidine auxotrophs to

autotrophs. In this test, bacterial strains are used having mutated histidine locus. As such, they do not synthesize histidine and thus, die when plated on an agar medium lacking histidine (Ames et al., 1975; Mortelmans and Zeiger, 2000). However, compound/NMs will enable the bacterium to synthesize histidine due to the reversal of mutation in histidine gene. The bacteria form colonies in minimal histidine medium. Bacterial cell wall can be modified with deep rough (RFP) mutation, which eliminates the polysaccharide side chains of lipo-polysaccharides, to make the bacteria more permeable. As the bacterial cell wall is rigid and semi permeable, it allows only a few NMs to cross the cell wall. Hence, to increase the suitability of the Ames test for NMs, this modification can be adopted.

Different assays such as the gene mutation assays hypoxanthine phosphor-ribosyltransferase (HPRT), comet assay, phosphatidylinositol glycan, thymidine kinase, Class A (Pig-a), chromosomal aberration test and micronucleus assay, can be adopted in mammalian cells (either cell lines or primary cultures) to assess the ability of NMs to induce various kinds of DNA damage (Chen et al., 2014; He et al., 2008; Shinohara et al., 2009). The genotoxic potential of NMs is then finally established using *in vivo* studies.

V79 Chinese hamster cells can be used to assess the HPRT forward mutation assay. This test assesses of the genotoxicity of a substance (Finette et al., 2002). The cell lines used have one functional copy of the HPRT gene located on X-chromosome. This gene is involved in phospho-ribosylation of hypoxanthine and guanine. A toxic analog of guanine, i.e., 6-thioguanine is added in the media and cells are grown in this. This poisonous 6-thioguanine is incorporated in DNA duplex during replication by HPRT enzyme leading to cell death. However, if the compound or

NMs induces any mutation (spontaneous and induced) in the HPRT gene, the toxic 6-thioguanine will not be incorporated during the DNA replication process as the salvage pathway does not function properly. Thus, the number of visible colonies represents the frequency of deleterious point mutations. Studies with different NMs have shown largely negative results (Chen et al., 2014).

The micronucleus assay is based on the scoring and comparison of the micronucleus. This method is faster and easier than the chromosomal aberration test. This assay is broadly used to assess the carcinogenic and genotoxic potential of the NMs. Micronucleus is a chromatin-containing structure formed from the lagging chromosomes or their fragments during the anaphase stage of cell cycle. It is present in cytoplasm surrounded by a membrane without any detectable link to the nucleus. In this assay, cell division is inhibited by a cytokinesis blocking agent (cytochalasin-B) which gives a binucleated appearance to the cells. This enables a more accurate scoring by reducing the incidence of false positives. However, the counting of micronucleus is hindered at higher concentrations of NMs due to deposition on the cell surfaces(Dobrzyńska et al., 2014; Li et al., 2012; Magdolenova et al., 2014; Shukla et al., 2013a).

Another technique used to detect the single- and double-stranded DNA break in individual cells is Comet assay. It is a rapid, simple, sensitive and frequently used technique. It is used to detect oxidative DNA damage, a basic sites, DNA--DNA or DNA--protein cross-links and quantification of alkali-labile sites. It also detects the damaged bases by incubating nucleoids with lesion-specific endonucleases, such as endonuclease III (Endo III) and formamidopyrimidine DNA glycosylase (FPG) that recognize oxidized pyrimidines and purines,

respectively (Karlsson et al., 2009; Shukla et al., 2011; Stone et al., 2009). Singlecells are suspended in low melting point agarose and spread onto a normal melting agarose microscope slide to make a monolayer of cells. The cells are then sandwiched with another thin layer of agarose to prevent loss. These cells are then subjected to alkaline lysis to obtain nucleoids, which then undergo alkaline electrophoresis. After electrophoresis, the neutralization step allows some renaturation of the DNA, and the DNA is stained with a fluorescent dye-ethidium bromide. Cells with higher DNA damage display increased migration of chromosomal DNA from the nucleus toward the anode, which resembles the shape of a comet when viewed under a fluorescent microscope. A qualitative and quantitative assessment can be done by using commercially available software. Moreover, the presence of NMs in the comet head (nucleoid) interferes and induce additional DNA damage (Karlsson, 2010). It has also been found that NMs and ions released due to the dissolution of the particles interact with FPG enzyme leading to an inhibition of enzyme activity which hampers the detection of oxidatively damaged DNA in the comet assay (Kain et al., 2012). The inhibition can be justified by the fact that ions are getting bounded ions to the --SH groups at the active site or due to physical hindrance by NMs. A more precise tool to sense the double-strand breaks is the analysis of γ-H2AX, one of the components of nucleosome core histone H2A family. The phosphorylation of this protein at serine-139 is mediated either by ataxia telangiectasia mutated, ataxia telangiectasia, and Rad3-related protein or DNA-dependent protein kinase leading to the formation of γ-H2AX, which is present in a complex form in the cell, and DNA double-strand breaks activates its phosphorylation. This alters the complexes into monomers which are thought to act as signals to recruit and retain DNA repair proteins to the DNA double-strand breaks site. The alteration in the expression profile of γ-H2AX induced by

ENPs has been detected by different techniques such as immune-histochemistry, flow cytometry, and Western blot (Ismail et al., 2007; Lewis et al., 2010).

To identify the mechanisms involved in carbon-based NM mediated toxicity in cells a method of mechanistically identifying the effects in both prokaryotic and eukaryotic cells was described by Riding et al. It utilizes the multi-beam synchrotron radiation-based Fourier-transform infrared imaging at diffraction-limited resolution and overcomes many of the intrinsic difficulties of assaying nanotoxicity and demonstrates exceptional sensitivity in identifying the effects of NMs in cells at environmentally-relevant concentrations(Riding et al., 2012).

5. Characterization of Nanomaterials for toxicological evaluation

5.1. Characterization of nanomaterials in biological matrices

The behaviour of NPs in the biological system greatly depends upon its surface characteristics. On the other hand, NMs require widespread characterization, unlike chemical compounds, and it is not limited to chemical composition or purity determination. This is because of the reason that the precise properties of NPs and their correlation with its biological activity are inadequately understood. As a result, an additional widespread and complete characterization including surface area, surface chemistry, size distribution, shape, porosity, agglomeration state, crystallinity, surface charge, solubility, *etc*., is strongly suggested for NMs characterizations in order to conclude the accurate correlation among their physicochemical properties and the biological effects elicited by them. In all of the parameters - that must be taken into account for characterization - size is the most significant as well as a vital aspect for determining the NPs interactions with living systems. Proper characterization leads to a better understanding and

greater reliability of results (Berhanu et al., 2009; Powers et al., 2007; Sayes and Warheit, 2009; Warheit, 2008). Additionally, the characteristics of NPs available commercially and specified by the manufacturer occasionally vary from those established by the researcher (Sayes et al., 2007). A change in activity is also observed between laboratory synthesized NPs and industrial scale manufactured NPs. Nevertheless, as the amenities in most of the research laboratories are not fully inclusive, the absolute characterization of NPs is often not easy. In the non-existence of a sophisticated laboratory unit with the entire instrumentation and experienced manpower requisite, researchers are bound to exploit the *modus operandi* accessible to them. Thus, occasionally, it is the accessibility of amenities that established the type of *modus operandi* for characterization to be executed than the experimental design or study needs.

A diversity of techniques are available to determine the size of NPs, and the most frequently utilized *modus operandi* are dynamic light scattering (DLS), Brunauer--Emmett--Teller (BET), transmission electron microscopy (TEM), atomic force microscopy (AFM) and scanning electron microscopy (SEM). Though, a further challenge that arises at this point is the divergence among size distributions and average sizes obtained by alternate methods. This is apparently not astonishing in consideration of the diverse basic principles behind the techniques implicated. Additionally, deviations in sample preparation scheme and apparatus operational procedures also add to measurement dissimilarities. Though, this possibly will initiate misunderstanding regarding the concrete size and size distribution of NP if the operator is not experienced in the principles and practical details of the measurement techniques concerned, as is repeatedly the case.

The US National Institute of Standards and Technology (NIST) have formed the world's first reference material standards (RMS) of gold NPs for nano-research. These gold NPs are present in three sizes: 10, 30, and 60 nm. They have been comprehensively analyzed by NIST for NP size distribution and size and by multiple techniques, and detailed measurement protocols including the data achieved are incorporated in a report accompanying all the standards. These RMS are mainly proposed for estimating and qualifying techniques and/or instrument performance among the dimensional/physical characterization of NPs. Additionally, they may be applicable for the development and assessment of *in vitro* assays that are intended to analyze biological responses to NPs, and for use for the inter-laboratory comparisons (Maddinedi et al., 2015).

When it comes to the NP toxicity; NP surface area is an important factor, because the NP and biological systems interaction takes place at their surfaces. The BET method is characteristically used to determine the solids' surface areas through the gas molecules' physical adsorption onto the solid surface. It includes adsorption a liquid nitrogen monolayer on the particles' surfaces thereafter estimating the quantity of nitrogen unconstrained upon vaporizing that layer. Therefore, the BET surface symbolizes the surface area which is generously reachable to gas molecules. The diameter of a primary particle (supposed as the corresponding sphere diameter) is further calculated from the precise surface area and the particles density - data is already available for the protocols. The advantages of this technique lie with the fact that it can afford two parameters at the same time, that is, surface area and size. But an associated pitfall is there that presupposes average-sized spheres containing monodisperse system, so it does not give

explanation regarding the particles' size distribution, which is the main parameter in toxicity evaluation with size-dependency (Powers et al., 2007; Weibel et al., 2005).

Electron microscopy is the easiest and most extensively used *modus operandi* that directly measures size distribution, size, and morphology for materials. On the other hand, it is time taking and needs enough number of materials containing the fields to be studied prior to a proper statistical appraisal can be completed. Furthermore, it examines materials in a dry appearance, not in the form of suspension, and needs the vacuum drying of samples, which may modify their properties. An additional disadvantage of this method is that it is unable to determine the particles' properties in the dispersion form, which is used for investigational revelation (Powers et al., 2007).

AFM is a cost-effective tool with a number of advantages for NPs characterization. It uses a cantilever along with an extremely slim probe to swing over the sample surface. It offers 3D visualization along with perpendicular resolutions of below 0.1 nm and X--Y resolutions of approximately 1 nm. For individual NPs, it gives information on several physical properties: surface texture, size, roughness and morphology (Gupta et al., 2005). Unlike other microscopic *modus operandi* where the statistics are feeble, AFM gives an alternative for accomplishing superior statistical significance by having numerous scans. TEM/SEM investigation is normally performed in vacuum, while the characterization of NPs by AFM can be achieved in ambient air and in liquid dispersions, which could be exceedingly helpful for biological studies. AFM examination also recommend a wider range of NPs from 1 nm to 8 μm and is able to calculate

with a single scan (Scalf and West, 2006). Furthermore, it involves lesser laboratory space than TEM/SEM and is is simpler to function.

DLS measures time dependent fluctuations in scattering intensity produced by particles in Brownian motion, and yield the size of the particle by applying the Stokes--Einstein relation. The size obtained by DLS is usually greater than that measured by other techniques, like TEM, BET, *etc*. This can be attributed to the fact that DLS measures Brownian motion and the subsequent size distribution of an ensemble of particles in solution there by yielding the mean hydrodynamic diameter, which is usually larger than the BET or TEM diameter as it includes a few solvent layers (Hradil et al., 2007). Throughout, in DLS measurements, there is a tendency of NPs to aggregate in the aqueous form, so it provides the sizes of cluster NPs than single NP. It gives an intensity weighted average hydrodynamic diameter of a compilation of NPs, so any sample polydispersity will skew the average diameter in the direction of larger NP sizes (Dhawan et al., 2009). This method gives additional feature for the alternative of considering the average hydrodynamic diameter of the NPs in terms of number. Considering the NP size in terms of both number and intensity might include value to the investigation. It can calculate the hydrodynamic diameter under circumstances that closely resemble the exposure surroundings, so it might give an idea for the NP stability in suspensions relating to the medium and time. Murdock et al. demonstrated the effectiveness of DLS by analyzing the reliance of the *in vitro* toxicity estimation on the dispersion state, the medium of exposure, the serum presence, the time gap among exposure and sample preparation (Murdock et al., 2008). It is an assembly method where the amount of a compilation of NPs is used to estimate the size distribution.

Recent studies based on the Brownian motion of NPs are called as NP tracking and analysis (NTA). This allows NPs to be visualized individually with concurrent examination of their Brownian motion. The particle size distribution might be attained on a particle-by-particle basis, permitting higher resolution and consequently an improved appreciative of aggregation as compared to ensemble methods like DLS. It circumvents any intensity bias near large NPs that could consequence in a small number of large agglomerates/particles masking the existence of a large number of NPs, as noticed with other light-scattering techniques. NTA could be used to recognize and count NP agglomerates owing to its capability to visualize the NP independently (Montes-Burgos et al., 2010).

Examinations of NP surface structure and composition is usually not given the equal values as shape, size, agglomeration, *etc*. On the other hand, the role of the NPs' surface properties in their toxicity and how these properties are modified throughout exposure under the influence of diverse environments desires awareness, as they govern the way in which NPs interact with bio-environments. Electron spectroscopies (X-ray photoelectron spectroscopy and Auger electron spectroscopy(AES), secondary ion mass spectroscopy, AFM, and scanning transmission microscopy are a few surface analytical technique to give information regarding elemental composition, topography, molecular and chemical state, structure (Baer et al., 2010). A thorough evaluation of these methods and the technical challenges encountered to apply these surface analysis tools to NP characterization was made by Baer and his co-workers (Baer et al., 2010). In any type of characterization, a constant fine particles example is the first and most essential step. Samples for characterizing NPs and for successive toxicity studies are generally taken in small quantities (often mg), but they must be the representative of the whole sample. Diverse ways of

performing reliable powder sampling and some general error connected with sample preparation have previously been discussed in detail by Powers and his co-workers (Powers et al., 2007). The NPs properties in liquid suspensions be liable to alter the surrounding environment and with time. NPs physical properties former to exposure may alter once they are in the cellular environment, again placing the stress on characterization at diverse investigational steps.

Though, the choice of a particular characterization technique depends on the type of NP being examined and the ultimate application of the NPs, it is suitable to execute multi-technique analysis so as to get a broader perception and more dependable photographs of the NPs characteristics. Association among many laboratories which possess expertise in their relevant methods need to be encouraged. A sufficient number of NPs should be calculated to get statistical significance.

5.2. Problems associated with measurement and characterization of nanomaterials.

Although there are number of characterization techniques available, but still some shortcomings complicate the development of methods used to identify NMs used in food and other biological matrices. The first thing which needs to be identified is whether the NMs are naturally present in it or intentionally added to food matrices (Morris, 2011; Ostrowski et al., 2015). Sometimes, the naturally present NMs are often mistaken for intentionally added NMs. The NMs in dairy products are mostly comprised of colloids, emulsions and biopolymeric NPs even before the processing steps have been applied (Nandita et al., 2015). Traditional manufacturing steps such as grinding and spray-drying are also reported to produce NMs of the natural ingredients. The NMs thus produced by this method are potentially non-toxic in nature. Differentiating these NMs

from the intentionally added NMs is a challenge for method development for risk identification and management. Another challenge is that NMs undergo different types of physicochemical changes during processing, manufacturing, packaging, consumption and absorption (Li and Huang, 2008; Stark, 2011). Inorganic NMs, in their initial pure form, tend to have similar characteristics at the manufacturing unit, which is easier to identify, characterize and quantify. Once these NMs are exposed to food matrices, some changes are conferred upon to both NMs and the food material. These changes include agglomeration or aggregation, change in shape, chemical form, surface chemistry, solubility/dispersibility, porosity and chemical reactivity. Changes in size and shape greatly alter the properties making it difficult to use a single method for characterization. The degradation and disintegration process in gastro-intestinal tract greatly reduce the size of solute materials, increasing its reactivity and bioavailability. Thus, for developing characterization method, the changes in NMs properties from manufacturing units up to the absorption of these NMs have to be considered (Alfadul and Elneshwy, 2010; Arora and Padua, 2010).

The most commonly used NMs include titanium, silver, silicon, zinc, iron and calcium. These NMs may be present singly or in combination with other inorganic or organic NMs such as lipids, proteins, and polysaccharides. When a mixture of NMs is used, then specific combination of techniques for characterization has to be used. NMs also have different textures ranging from hard metals to soft nanoemulsions or nanoliposomes. For developing characterization methods, one also has to keep in mind about the purpose of NMs usage in food. NMs, whether natural or manufactured, has been used for a variety of purposes including increasing shelf life, appearance,

rheology, stability, texture or for organoleptic characteristics. Thus, a variety of analytical approaches is required to get desired decision outcomes (Hwang et al., 2012; Yada et al., 2014).

Prior to characterization techniques, the sample (food matrices or biological material) has to undergo extraction process to "release" the NMs. Incorporation of NMs to food matrices also alter its reactivity. Thus, a single extraction method is also not applicable for all types of food products. The pre-treatment process may also involve any change to NMs used and may vary the results. A standardized protocol, catering to the changes involved through the process of manufacture and consumption, has to be developed for desired output (Bandyopadhyay et al., 2013).

6. Nanomaterials in Food and its toxicity

New technologies offers significant benefits to human, however they possess multiple risks to human and environmental health. NPs could enter the food chain via routes including nutrients, pesticides, environmental pollutants or through processed foods(Rico et al., 2011), raising concerns of toxicity in the ecosystem. Therefore, detailed life cycle analysis, particle uptake by plants, bio-distribution, entry in the food chain, *etc*. need a thorough investigation for these tools are used as products in agri-food sector. A variety of factors have to be taken into consideration before the impact of NP exposure on human health (Jasmine et al., 2010). Initiatives leading to better understanding and acceptance of the NP based products are needed for technology development. The evolution of a participatory, dynamic and responsive nanotechnology policy and coordinated risk management strategy for the Indian agriculture and food system would be needed if the positive economic impacts of nanotechnology are to reach

the agrarian society (Kalpana et al., 2013, 2010). The small size and successive larger surface area of NPs endows them among some extremely valuable and precise properties but, it also gives them biologically more active leading to unpredicted and unexpected consequences on interaction with biological structures. Smaller size also conveys a dissimilar bio-kinetic behaviour and capability to reach extra distal sections of the body (Oberdörster et al., 2005). The work-related introduction of NPs will also amplify with the growing production and use of NMs socially.

Environmental contamination is hitherto an additional apprehension. These apprehensions have generated concerns about the probable undesirable effects of engineered NMs upon the human and the environment healthiness. Government, regulatory authorities and scientific authorities for Environmental, Health and Safety all over the world are realizing the importance of NM risk assessment. Figure 2 depicts the interlinked different factors for determining environmental and health risks due to engineered NM exposure.

A systematic knowledge of the mechanism of NPs inflowing and out-flowing the cells could also direct to a enhanced understanding of NP toxicity including enhancement in their bio-medical applications. This will enable the formulation of regulatory rules to reduce the risks involved in the field. The European Commission's Scientific Committee on Emerging and Newly Identified Health Risks (SCENIHR) has looked into the existing information/data and problems to be considered in conducting risk assessment on NMs (Dasgupta et al., 2015a; Ranjan et al., 2014). The European Commission's Scientific Committee on Consumer Products (SCCP) released an article entitled "Opinion on Safety of NMs in Cosmetic Products." They

raised their worry concerning huge data gaps, inappropriateness of existing methodologies for NP risk evaluation and insufficient information about NPs on skin amalgamation in both abnormal (diseased) and normal skins. Regulatory documents on safe handling of NMs are also being outlined by different scientific groups (Dhawan et al., 2011, 2009; XpertArena, 2015).

6.1. Usage of nanomaterials in food

Nanotechnology is also administered into the 'food sector' which includes nanosensors, tracking devices, targeted delivery of required components, food safety, new product developments, precision processing, smart packaging *etc*. (Huang et al., 2010; McClements et al., 2009; Yu and Huang, 2013). Nanostructured materials exhibit unique physicochemical properties that open windows of opportunity for the creation of new, high performance materials, which will have a critical impact on food manufacturing, packaging and storage. Currently, the application nanotechnology in food production chain is focused on the development of nano-sized food ingredients and additives, delivery systems for bioactive compounds and innovative food packaging. Additionally, the applications such as a nanocoating that protects tomatoes from humidity and oxygen, bread containing nanocapsules of omega-3 fatty acids and juice containing vitamin A encapsulated in starch, use of nanocans for packaging and transportation of liquid beverages because of their light weight are gaining importance by the consumers (Cushen et al., 2012; Ravichandran, 2010).

Natural protein, carbohydrate and fat molecules have been modified with nanotechnology and the modified forms are being used in food packaging and food ingredients including food additives, nutraceuticals *etc*., but the long term focus can be brought upon controlled release of

nano-encapsulated food ingredients or nutrients (Dasgupta et al., 2015a; McClements, 2015). Nanotechnology can also improve the water disperseability, thermal stability and oral bioavailability of the functional compounds of food (Dasgupta et al., 2016; McClements et al., 2009). Various applications of NPs in the food industries are globally focused on: (i) sensory improvements (flavour/colour enhancement or texture modification), (ii) increased absorption and targeted delivery of nutrients and bioactive compounds, (iii) stabilization of active ingredients such as nutraceuticals in food structures, (iv) packaging and product innovation to increase shelf life, (v) sensors to assess the safety of food, (vi) as an antimicrobial agent against the food born pathogenic bacteria. The stability of NMs in food is dependent on a range of storage conditions at varied temperature. This may affect both, the stability of NPs within the food; as well the change in the properties of the biomolecules after their interactions with the NPs (Dasgupta et al., 2015a; Monica and Calster, 2010; Ranjan et al., 2016; Selin, 2007). The application of nanotechnology to the food sector may allow the modification of numerous macroscale characteristics of food such as texture, taste, other sensory attributes, colouring strength, processability and stability during shelf life which helps to increase physiochemistry of food.

6.2. Measurement of nanomaterials in food and other biological matrices

NMs vary in their size and shape and also may undergo modifications during processing and manufacturing units. Once ingested, NMs also interact with different biological materials. Although some characterization techniques are present but no single method is applicable to all the NMs to predict the safety for consumption. The different physical and chemical properties

also make it difficult to develop a single characterization method. Thus, a combination of varying techniques can be employed to predict potential benefits or risks (Kettiger et al., 2013; Kunzmann et al., 2011; Magnuson et al., 2011). With the help of the current available techniques, it is now possible to identify if NPs are present or not in biological matrices. Inorganic NMs, primarily silver, gold, and silica NPs have the most established detection techniques including flame atomic absorption spectroscopy (Karimi et al., 2011), surface plasmon resonance (Jeong et al., 2015) and inductively coupled plasma technology (ICP) coupled with either mass spectrometry (MS), atomic emission spectroscopy (AES), or optical emission spectroscopy (OES) (Fabricius et al., 2014; Quarta et al., 2012). For specifying combination of methods from the above mentioned techniques, one has to list out the objective for which the characterization has to be done. It is solely to find out if the NMs are present or not; or the changes incurred upon the biological matrices after interaction with NMs. Detection methods can also be used to analyze the commercially available products to find if the NMs added have changed its properties similar to its bulk counterpart during the processing or have aggregated to change its size and shape. Electron microscopy can be employed to identify modification in size, shape and porosity. Apart from its size and shape NMs' chemical composition also affects its properties and the extent of translocation of these NMs from the gastrointestinal tract to different organs *via* blood. Inorganic NMs, if present in their ionic form, are reported to be more toxic than its stable form. However, if the surface chemistry has been modified or changed, then other more specific techniques are required.

Current techniques are more efficient in quantification and measuring the properties of inorganic NMs, however, the same cannot be said for organic NMs comprising of proteins, lipids,

polymers and polysaccharides which resemble the biological materials. Elemental NMs are easier to be detected and quantified than the organic ones. To detect any NM from a given biological sample such as food matrix or intestinal cells, the sample must be digested to release the free NMs. Once extracted, some of the aforementioned techniques may be utilized to determine the presence of NMs. Enzyme-linked immunosorbent assay (ELISA) kits for antibody-based detection and flow cytometry are the rapid screening techniques which may be useful for detection of organic NMs (Dehalu et al., 2012). The mixed NMs, that is, inorganic core with organic coating, needs a set of paired techniques, mainly electron microscope combined with sample chemistry based methodology e.g. X-ray photoelectron spectroscopy, scanning probe microscopy, scanning transmission microscopy, AFM, low energy iion scattering technique, and secondary electron mass spectroscopy (Baer et al., 2010; Kettiger et al., 2013; Magnuson et al., 2011).

Many companies consider these advanced instrumentations too costly and time-consuming in comparison to other techniques like high-performance liquid chromatography (HPLC) and DLS. Recently researchers have standardized the HPLC characterization for nano-encapsulated food products and the same have wide opportunities (Nandita et al., 2015). Due to the several challenges, attaining informative data from complex materials methods have not been well-validated for characterizations of organic and inorganic NMs in food and drugs (Corredor et al., 2015; Wise and Brasuel, 2011). For example, chemical imaging techniques and electron microscopy techniques provide NMs image data successfully when the samples have large changes in disparity (chemical as well as optical respectively, or both) between the NMs and the surrounding matrices. This is creating a big challenge to locate NMs such as carbon nanotubes

within cells and tissues rich in carbon. Moreover, a complication created in the sample preparation methods because of labile behaviour of NMs can ultimately results in the image data that cannot be distinguished between concepts like engineered NMs migration versus its agglomeration. Labelling organic NMs *via* fluorescent tags or radiolabels may be a potential troubleshoots for some of these issues. However, such modifications may change the chemical or physical characteristics of organic engineered NMs and make them poor models for their unlabeled versions. It is a major challenge in the research to develop reliable methods for imaging NMs in food matrices and alimentary tract cells/tissue than those based on present detection technologies. Researchers need to develop new analytical approaches for organic NM sampling, detection, and quantification, as well as imaging of both inorganic and organic NMs. Additionally, it is needed to assess the hazards of NMs in food, drug, food/drug contact materials and the alimentary canal (Alger et al., 2014; Ostrowski et al., 2015).

7. Nanoparticles mediated alterations on major organ systems

7.1. Exposure to Nanomaterials

Every day we are exposed to a number of NMs, whether anthropogenic or natural. The manufacturing units of NMs also pose a threat of exposure to humans or environment. Spilling or effluent discharge from industries or research labs add up further contamination. Another direct source of exposure is through cosmetics, personal care products or food through different routes such as inhalation, digestion or dermal exposure and the washing off of these consumer products results in entry of NMs into the environment (Mihranyan et al., 2012).

Inhalation is one of the most common route of exposure to NPs (Bakand et al., 2012)**.** The large scale production of powder during NMs synthesis, processing and/or packaging also possess a serious risks to the workers engaged in these activities. Lack of regulatory checks on the manufacturing units also enhances to the chance of leaking of NPs to the environment. These air borne NPs pose a lethal effect to the health through the respiratory system (Jasmine et al., 2010; Kim et al., 2009). Any overseas particle inflowing towards respiratory tract can induce toxicity mainly in three regions - nasopharyngeal, trachea-bronchial, and alveolar regions and also face several clearance mechanisms especially in epithelial and alveolar macrophages. Alveolar macrophages can efficiently phagocytize clusters of fine and coarse particles but not for singlet NPs which can then translocate to interstitial sites and to regional lymph nodes. Through the blood circulation they can then be dispersed to another organs *e.g.* liver and spleen (Dhawan et al., 2011). These particles are then either eliminated out or are retained within the body and again translocated to other organs. Likewise, exposure of carbon based NMs -- mainly CNTs -- results in platelet aggregation, aortic DNA damage and enhanced vascular thrombosis through inflammatory events which results in adverse cardiovascular effects.

Dermal exposure is another exposure route for the NMs entry. The NMs barrier is still to be completely explored. Trans-appendageal, inter-cellular and trans-cellular can be possible routes for NMs (Wu et al., 2013; Yan and Chen, 2013). The NMs soluble in lipid may move through lipid rich membranes among skin cells inside the intercellular routes, while, and within the transcellular route the substance penetrate the skin cells. The hair follicles and sweat glands spread all around the skin in various densities may become the means for NMs entry for the trans-appendageal route (Albanese and Chan, 2011; Crosera et al., 2009; Love et al., 2012)**.**

Direct ingestion of NMs occurs when they are used in drug delivery, food, food packaging and cosmetics. Apart from these, effluents from the manufacturing units or discharge from the consumer products directly enters the environment. Since, removal of these NMs from the discharge is very difficult, they can potentially enter into the food chain and these swallowed NPs can possibly be translocated *via* the lumen of the intestinal tract into several organs (Pietroiusti et al., 2013). In a study by Bockmann et al (2000), translocation of TiO_2 NPs to different organs through the GI tract *via* the blood has been reported (Böckmann et al., 2000). The extent of uptake is also dependent upon size and shape of the NPs. Triangular shaped NPs are found to be more toxic than spherical NPs (Chan et al., 2008; Dasgupta et al., 2015b; Huang et al., 2007). Kidney, being a chief excretory organ, also gets influenced by any kind of direct or indirect injuries caused by NP mediated toxicity. In a study, 6.6% of the administered 50 nm particles, 5.8% of the 100 nm particles, 0.8% of 1 µm particles, and 0% for 3 µm particles of polystyrene particles were found to be translocated from the Pyer's patches into the mesenteric lymph and then to systemic organs (Jani et al., 1990). Such effects of NPs on different organ systems have been discussed in detail in the following sections of the paper.

7.2. Nanoparticles mediated cardiovascular alterations

NPs exposure has been correlated to the cardiovascular diseases onset and progression in different studies and has become a key concern in the fields of nanotoxicology and cardotoxicity. These studies point towards a strong association between NP exposure and cardiac alterations (Pope et al., 2004). NPs primarily induce endothelial dysfunction that ultimately leads to pathological complications in cardiovascular system by development of atherosclerosis, acute

coronary syndrome and myocardial infarction (Figueira et al., 2013). To test the hypothesis of endothelial dysfunction, iron oxide NPs were studied to evaluate their probable risks on human endothelial system and the effects on human aortic endothelial cells as well as monocyte mediated effects by phagocytosis were investigated. Phagocytosis and dissolution of NPs by monocytes were found to simultaneously initiate oxidative stress leading to severe endothelial toxicity thereby inducing downstream cardiovascular problems (Zhu et al., 2011). Several studies have evaluated the effect of inhaled and intra-tracheally instilled CNTs on cardiovascular system. Intrapharyngeal instillation of single walled CNTs (SWCNT) on mice showed activation of heme-oxygenase-1, a marker of oxidative stress, in lungs, aorta, and cardiac tissues. Aortic mitochondrial DNA damage was observed with changes in mitochondrial glutathione levels and protein carbonyl levels. Atherosclerotic plaque formation was observed in response to increased platelet activation which may lead to other chronic inflammatory responses in the tissue (Li et al., 2007). Inhaled CNTs have also shown the disruption of physiological homeostasis in heart and vasculature resulting in altered autonomic cardiovascular control regulation (Legramante et al., 2009). These studies clearly suggest a causative association between CNTs exposure and deleterious alterations in cardiovascular disease progression. Mechanistic pathways involved in CNTs mediated cardiotoxicity were characterized in different *in vitro* studies and showed that actin filament vascular endothelial disruption leads to the translocation of CNTs into the systemic circulation (Helfenstein et al., 2008; Walker et al., 2009). Future studies are required to investigate the potential of these CNTs to exaggerate the systemic inflammation and establish the signalling pathways for the initiation and progression of cardiovascular diseases.

In vitro and *in vivo* evaluation of silica NPs was done on zebrafish to study the cardiovascular effects and it was observed that they induce cytotoxicity, oxidative stress and apoptosis leading to endothelial cells dysfunction and ultimately cardiovascular alterations. Silica NPs cause pericardial toxicity and bradycardia *in vivo* and inhibit angiogenesis. These alterations discomfit the heart formation and development and serves as possible risk factors for cardiovascular system (Duan et al., 2013). Unlike chemical drug molecules, engineered NPs are not generally tested for cardiotoxicity in the clinical trials. Several NPs are used routinely in clinical applications and have a potential risk to interact with cardiac cells and alter the normal functioning. Above mentioned studies forcefully indicates that there is an utmost need of studies linking exposure of NMs and cardiovascular diseases.

7.3. Toxic effects of nanoparticles on the respiratory system

The most common route of NP entry in body is through inhalation via respiratory system. Respiratory system gets affected by two different mechanisms, directly by the inhaled NPs in the respiratory tract and indirectly by the deposition of NPs in lungs via blood circulation. Direct entry of NPs primarily affects nasopharyngeal, tracheobronchial and alveolar regions. Particles with smaller dimensions are expected to be more vicious to the lung than the larger particles. Also, the particles with more inert surfaces may also be aggressive and can exert their effects on cells because of the large surface area (Donaldson et al., 2006). Foremost threat with the NPs entering in the respiratory tract is that they can enter the bloodstream through alveoli and affect other organs. Inhaled NPs gets deposited in the alveolar regions and play a central role in

pulmonary toxicity as they leads to a dispersed chemo-attractant signal and suppress the alveolar macrophage response (Kreyling et al., 2002).

Acute toxicity of silver and carbon nanoaerosols on normal and cysticfibrosis human bronchial epithelial cells was studied and it was observed that patients with chronic airway diseases are more prone to the adverse effects of nano sized particulate matter present in air pollution (Jeannet et al., 2015). Biokinetic studies have shown that inhaled NPs can translocate via olfactory neurons through the nose to the central nervous system (Oberdörster et al., 2004). Major respiratory effects of NPs comprise peribronchial inflammation, multifocal granulomas, progressive interstitial fibrosis, collagen deposition, chronic inflammatory responses and oxidative stress (Ferreira et al., 2013; Shvedova et al., 2009).

The response of respiratory system to CNTs has been evaluated in *in vivo* pulmonary models in different studies and potential respiratory risks were reported. Intratracheal instillation of different SWCNT samples on mice was shown to induce dose-dependent persistent epithelioid granulomas peribronchial inflammation in lungs as well as necrosis extending into the alveolar septa (Lam et al., 2004). Intrapharyngeal aspiration of purified SWCNT on mice resulted in acute inflammation, early onset of formation of granulomas, and progressive fibrosis. These granulomas were mainly associated with hypertrophied epithelial cells and diffusive interstitial fibrosis with alveolar wall thickening. Lung lesions were found to be dose-dependent and progressive. Different biomarkers of inflammation, oxidative stress, and cytotoxicity were also shown to be severely affected (Shvedova et al., 2005). Silica particles and ultrafine carbon black have shown early inflammation followed by the altered profile of bronchoalveolar lavage fluid.

Increased TNF-α and IL-1β expression in response to oxidative stress was also evident in the study (Warheit et al., 2004).

Multi walled CNTs (MWCNT) also showed inflammation, collagen rich granulomas, and fibrosis in the lung tissues of rats. Hydroxyproline and soluble collagen levels were also increased in a dose-dependent fashion (Muller et al., 2005). In a MWCNT acute inhalation study on Wistar rats, pulmonary inflammogenicity was observed upon the concentration-dependent MWCNT exposure and showed regression over time (Ellinger-Ziegelbauer and Pauluhn, 2009). In a 90-day inhalation toxicity study with MWCNT on Wistar rats, marked multifocal granulomatous inflammation, diffuse histiocytic and neutrophilic inflammation, and intra-alveolar lipoproteinosis were observed in lung and lung-associated lymph nodes (Ma-Hock et al., 2009). The impact of metal impurities present in some MWCNTs cannot be underestimated in the forms in which the lung could be affected and should be tested properly prior to their application on humans (Trout and Schulte, 2010). Intertracheal instilled ferric oxide NPs have shown to induce different clinical pathological changes *in vivo* including follicular hyperplasia, pulmonary capillary vessel-hyperplasia and alveolar lipoproteinosos in lungs (Shvedova et al., 2005; Zhu et al., 2011).Exposure level of different NPs increases the risk of developing pulmonary fibrosis in the population and should be critically monitored and controlled (Ashutosh and Alok, 2013).

7.4. Nanoparticle mediated toxicity on other organ systems

In addition to the NP toxicity on heart and lungs, other organ systems are also insensitively affected and hampers the normal body functioning. This includes digestive system,

nervous system, kidney, liver, reproductive system, skin and altered immune responses of body. Carcinogenic potential studies of CNTs suggest that these structures are not risk free, and generally dependent on several other physico-chemical and biological characteristics, and also specific composition and type and size of impurities (Shvedova et al., 2009). This again limits the use of CNTs in therapeutic purposes. It has also been observed that MWCNT gets accumulated in the Kupffer cells of hepatic macrophages for longer durations thereby inducing acute liver toxicity (Deng et al., 2007). This limits the use of CNTs as nanomedicines or as a delivery vehicle of other drug molecules. Recently, silver naoparticles were shown to generate ROS and suppressing the redox system thereby causing cytotoxicity in human liver cells (Hussain et al., 2005; S. Kim et al., 2009; Piao et al., 2011).

NPs can be ingested directly into the GI tract through the intake of food, water or nano based medication with oral route of delivery and hence it is considered as an important and initial target of NP mediated toxicity. Researchers have reported acute oral toxicity of different types of NPs. Nano-titania toxicity was evaluated in mice and symptoms of alimentary canal function disorder were observed like loss of appetite, diarrhoea etc. in addition to the side effects on kidney, liver, heart and spleen(Wang et al., 2008a). Zinc oxide NP treated mice showed inflammation in gastric lamina propria and submucosa layers.

Central nervous system acts as a critical target organ for NP inhalation or intranasal instillation exposure. It is affected by the NPs that are tranclocated by extrapulmonary route from respiratory tract to the nervous system in cases of acute exposure to NPs (Nemmar et al., 2001). Ultrafine NPs deposited in the olfactory mucosa also cause neurotoxicity (Elder et al., 2006).

Inhaled or intranasally instilled NPs can trigger proinflamatory responses in nervous tissues. Brain olfactory bulb have shown inflamtory alterations in response to 14 nm carbon black particles (Tin-Tin-Win-Shwe et al., 2006). Time dependent translocation of titanium oxide NPs from intra nasal to nervous system results in the deposition of NPs in the hippocampus and affects the neighbouring regions as well (Ranjan et al., 2015; Wang et al., 2008b).

NPs containing few specific transition metals have a higher potential to generate ROS, in addition to oxidative stress generated by inflammatory neutrophils and activated alveolar macrophages (Ellinger-Ziegelbauer and Pauluhn, 2009; Pauluhn, 2010). Systemic immune function alterations have been observed in mice in response to MWCNT as evident from the nonmonotonic systemic immunosuppression and decreased natural killer cell functions (Mitchell et al., 2007). In a study with SWCNT-transformed cells injected in immunodeficient mice, one week post-injection, tumours were found at the injection site in mice receiving B-SWCNT cells, whereas mice receiving control BEAS-2B cells did not show any tumours at the injection site indicating the potential role of p53 in this process (Wang et al., 2011).

A study was conducted suggesting that engineered NM exposure can lead to the microvascular impairments that persist throughout the multiple developmental stages. Microvascular and mitochondrial dysfunction were observed in the Sprague-Dawley female F1 generation after gestational nanosized titanium dioxide particle exposure where endothelium-dependent dilation in coronary and uterine arterioles were significantly impaired in addition to the reduction in maximal mitochondrial respiration in the uterus and left ventricle (Stapleton et al., 2014). The perseverance of this fetal microvascular dysfunction into adulthood may also

create the foundation for disease vulnerability, increase of rate of pathologies and/or toxicant sensitization.

Skin offers comparatively larger area for exposure to NPs and act as a major organ for the entry of NP into the body. NPs and NMs can penetrate the uppermost layer of skin called stratum corneum and access the viable epidermis and causes toxicity. This phenomenon of NP penetration is still under debate. It has been shown that oil-in-water emulsions of titanium oxide NPs can penetrate the skin surface through hair follicles and skin (Bennat and Müller-Goymann, 2000). Health risks related to silver NPs were evaluated for dermal toxicity, eye irritation, dermal irritation and corrosion and skin sensitisation. Dose dependent cytotoxicity of silver NPs were found to induce in microorganisms and mammalian cell lines (Kim et al., 2012). In an *in vitro* study, SWCNT have shown increase in cutaneous toxicity by increase in ROS in human epidermal keratinocytes (Shvedova et al., 2003).

8. Risk management of Engineered Nanoparticles

Unique properties that make NPs a beneficial technology for therapeutic and other important intervention may also lead to adverse health effects, hence, it is very important to determine, and appreciate, the fine balance between the efficacy and toxicity of NPs and NMs(Thorley and Tetley, 2013). Nanotoxicology risk assessment requires information about its capability to reach and react at the site of action, and the nature and magnitude of the resultant response at the site (**Figure 3**). The increasing concern leads to the growing need of technical requirements for the detection and characterization of environmental NPs and to drive the limits of modern sampling techniques and instrumentation (Dasgupta et al., 2016). *In silico* computational methods like

quantitative structure--activity relationship software analyse chemical reactivity, potential targets and bioavailability by structural similarity with substances with known such activities. Similarly, such programs can also be used in toxicology risk assessment of NPs to predict the potential physiological targets and downstream health effects resulting from human exposure to the NPs (Choi et al., 2013; Ranjan et al., 2015; Valerio et al., 2013). Direct and indirect toxicity of NPs should be tested using cell lines and different animal models for the assays including cytotoxicity, oxidative stress, and dose mediated responses, accumulation studies in different organ systems, inflammation and cellular death. NMs intended to be used in food products as well as of medicinal purpose should also be tested properly by *in silico, in vitro and in vivo* toxicity analysis (Dasgupta et al., 2015b; Lefebvre et al., 2015; Maddinedi et al., 2015; Ranjan et al., 2016). To maximize the potential of NPs in the field of medicine and food engineering, novel NMs should be rigorously tested in the labs.

Recent years have witnessed use of NMs in more than 800 consumer products including cosmetics, sunscreens, electronic components, ski waxes, cigarette filters, antimicrobial and stain-resistant fabrics, cleaning products, and self-cleaning windows. However, studies are also reported for its potential cyto- and genotoxic effects, inflammation and even cancer due to its large surface area to mass ratio. As the materials are in nano size, the physical properties are different from their bulk counterparts such as solubility, melting point, electrical conductivity, or changes in the crystalline structure of the materials (Elder et al., 2009; Savolainen et al., 2010)**.**

Regulatory authorities worldwide are realizing the risk associated with the usage of NMs. In 2003-June, the UK officials specially made The Royal Academy of Engineering and The Royal

Society to look into the benefits, safety and health related issues arising from the usage of NMs. The Royal Society published its report in 2004 entitled ''Nanoscience and Nanotechnologies: Opportunities and Uncertainties'' indicating the NPs or nanotubes must be treated as recent materials under the existing "notification of new substances" (NONS) rules as well as in the "registration, evaluation, authorization and restriction of chemicals" (REACH)' to set off further testing (Hirose, 2013; Jones and Grainger, 2009; Sharma et al., 2012b; Tervonen et al., 2009).

"United States Environmental Protection Agency" (USEPA) is also actively working in potential usage of NMs and the risks associated with it. It also stresses on development of NMs with a practical approach. Inside its document---EPA 100/B-07/001 (Nanotechnology White Paper) published in 2007, it has stated ''as the use of NMs in society increases, it is reasonable to assume that their presence in environmental media will increase proportionately, with consequences for human and environmental exposure''. Committees on the Toxicity, Carcinogenicity and Mutagenicity of Chemicals in Food, Consumer Products and the Environment have also identified the risk assessment of NMs as an area of interest in their 'Joint Statement on Nanomaterials Toxicology'.

The European Commission's Scientific Committee on Emerging and Newly Identified Health Risks (SCENIHR) has also reviewed the existing information/data and issues to be considered in conducting risk assessment on NMs (Sharma et al., 2012b). European Commission's Scientific Committee on Consumer Products (SCCP) issued a document titled ''Opinion on Safety of Nanomaterials in Cosmetic Products'' and raised a concern about large data gaps, inappropriateness of existing characterization techniques for NP risk assessment and inadequate

information regarding NPs absorption and uptake in both normal and diseased skins. Guidance documents on harmless management of NMs are also being outlined by researchers. Non-governmental organizations like “Friends of the Earth” and “Xpert Arena” have warned against nanotechnology in cosmetic and sunscreen products, since they may result in possible uptake of particles by human skin- if NPs penetrate the skin, they can join the bloodstream and circulate around the body with uptake by cells, tissues and organs leading to cause several diseases (Dhawan et al., 2011; Heinemann and Schäfer, 2009; Shivendu and Nandita, 2013; XpertArena, 2015).

9. Regulatory issues

Most of the countries don’t have defined regulations for the marketing and use of nano-derived industrial products. European policy is among those which tried to establish a strong and defined regulation for the same. Although the existing laws were considered for conventional food products, but, the same laws have been also considered on a broad aspect of nano-foods. Only recently, the European regulatory debate has been characterized by a change of perspective notably supported by the European Parliament, which in 2009 required that “the Commission review all relevant legislation within two years to ensure that legislative provisions and instruments of implementation reflect the particular features of nanomaterials to which workers, consumers and/or the environment may be exposed” - European Parliament Resolution on regulatory aspects of nanomaterials (Ramachandran, 2011). Consequently, the vast need of some regulations containing specific provisions addressing nanomaterials have made the entrance of laws/regulations. However, specific regulations do not exist for all food categories, therefore it is

quite difficult for food industry and private sectors to have clear guidance on the applicable regulatory framework (Marrani, 2013).Overall, there are only a few regulations providing specific provisions for NMs (Table 1). In spite of these few laws/regulations, the debate for the nano-regulation in the European institutions has raised over the time, which ultimately leads to the constitution of the EU soft law regarding nano regulatory framework (Table 2). It can be noted that, soft law is the term applied to EU measures, such as communications, resolutions, recommendations, opinions and guidelines, which, in contrast to directives, regulations and decisions (hard law), are not binding on those to whom they are addressed. Soft laws are also referred to as private laws since they are set following a stakeholder approach which maintains that the state, in its regulatory decisions, should be assisted by the private sector and civil society representatives, all involved in participatory decision processes. Literature and earlier studies on nano-food regulation identifies the incapability of the existing regulatory processes and keep a rapid policymaking and regulations are required for entering the marketplace (Chaudhry et al., 2010; Cushen et al., 2012; Dasgupta et al., 2015a; Marrani, 2013; Ramachandran, 2011; Ranjan et al., 2014; Shivendu and Nandita, 2013).

10. Conclusion

It can be summarized from the reported toxicological data that, the characterization of NPs is an essential criterion in determining the toxic potential of engineered NPs. It is essential to know more about the toxicological effects of NPs by considering a wide range of endpoints in order to deal with the increased concern of potentially harmful exposures. NPs related risks and hazards should be tested according to their potential routes within the human body in order to

study the detailed progression of particular diseases. Also, as most of the toxicology studies have done on animal models, there is a vital need for a strategy to extrapolate the toxicological data in biological systems to predict the risks and adverse outcomes in humans. Because of the flexibility in the physio-chemical properties of NPs and the vast complexity of hosting systems, there is a need to design advanced and highly accurate strategies to study the NP-cell interactions as well as the toxicity. Multidisciplinary techniques using different *in silico* to *in vivo* models and test methods should be used in computing the overall NPs associated side effects. With appropriate strategies that integrate risk assessment into decision-making frameworks for risk management with a better understanding and incorporation will fetch better results in the future and assist in designing environment-friendly and biologically safe NPs.

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Table 1: List for few of the European Union regulations providing specific provisions for nanomaterials (directly or indirectly)

S. No.	Name of regulations	Specific feature	Details (in brief)	References
1	Regulation (EC) No 1333/2008	For food additives	States that a food additive already authorized but obtained using nanotechnology requires a re-evaluation before marketing	(Marrani, 2013; Ramachandran, 2011)
2	Regulation (EC) No 1332/2008	On food enzyme	States that a food enzyme already included in the Community list but prepared by different methods or using starting materials significantly different (It is specified that "Significantly different" could mean a change in particle size) from those included in the risk assessment of the Authority, should be submitted for re-evaluation.	
3	Regulation (EC) No 450/2009*	active and intelligent materials and	Although nanomaterials are not directly mentioned, there is a reference to "substances deliberately engineered	

		articles intended to come into contact with food	to particle size which exhibit functional physical and chemical properties that significantly differ from those at a larger scale"; therefore, a case-by-case analysis has to be followed for active and intelligent materials and articles containing nanomaterials	
4	Regulation (EU) No 10/2011*	On "plastic materials and articles intended to come into contact with food"	States that substances in nanoform should be used only if listed in the annex I of the regulation.	

*It can be noted that, Both regulations 450/2009 and 10/2011 state the functional barrier concept which means it is not directly applicable to nano-materials

Table 2- Few main documents for European Union soft law for nano regulation

S. No.	Type of soft law document	Details	Reference
1	Resolution	European Parliament 2006, "Nanosciences and nanotechnologies: an action plan for Europe 2005-2009."	(Hellsten, 2005)
		European Parliament 2009, "Regulatory aspects of nanomaterials."	(Bowman et al., 2010; Maruszewski, 2014)
2	Communications	European Commission 2004, "Towards a European Strategy for Nanotechnology."	(Commission, 2004)
		European Commission 2005, "Nanosciences and nanotechnologies: An action plan for Europe 2005-2009."	(Commission and others, 2005; Hellsten, 2005)
		European Commission 2007, "Nanosciences and nanotechnologies: An action plan for Europe 2005-2009. First Implementation Report 2005-	(Maclurcan and Radywyl, 2011; Marrani, 2013; Ramachandran, 2011)

		2007"	
		European Commission 2009, "Nanosciences and Nanotechnologies: An action plan for Europe 2005-2009. Second Implementation Report 2007-2009	
		European Commission 2008, "Regulatory aspects of nanomaterials"	(Bowman et al., 2010; Maruszewski, 2014)
		European Commission 2012, "Second Regulatory Review on Nanomaterials"	(Schlyter, 2012)
3	Reccomendations	European Commission 2008, "Code of conduct for responsible nanosciences and nanotechnologies research"	(Dorbeck-Jung and Shelley-Egan, 2013)
		European Commission 2011, "Definition of nanomaterial"	(Lidén, 2011)
4	Guidelines and reports	EFSA 2011, "Guidance on the risk assessment of the application of nanoscience and nanotechnologies in the food and feed chain"	(Committee and others, 2011)
		EFSA 2013. Annual report of the	(Berton-Carabin and

		EFSA Scientific Network of Risk Assessment of Nanotechnologies in Food and Feed for 2013	Schroën, 2015; Savolainen et al., 2013)
		EFSA 2015 Annual report of the EFSA Scientific Network of Risk Assessment of Nanotechnologies in Food and Feed1 for 2014	
5	Opinions	Opinion of the European Economic and Social Committee on the Communication from the Commission: Towards a European strategy for nanotechnology, (2005)	(Macnaghten et al., 2005)
		Opinion of the European Economic and Social Committee 2009, "Nanomaterials"	(Grieger et al., 2009)

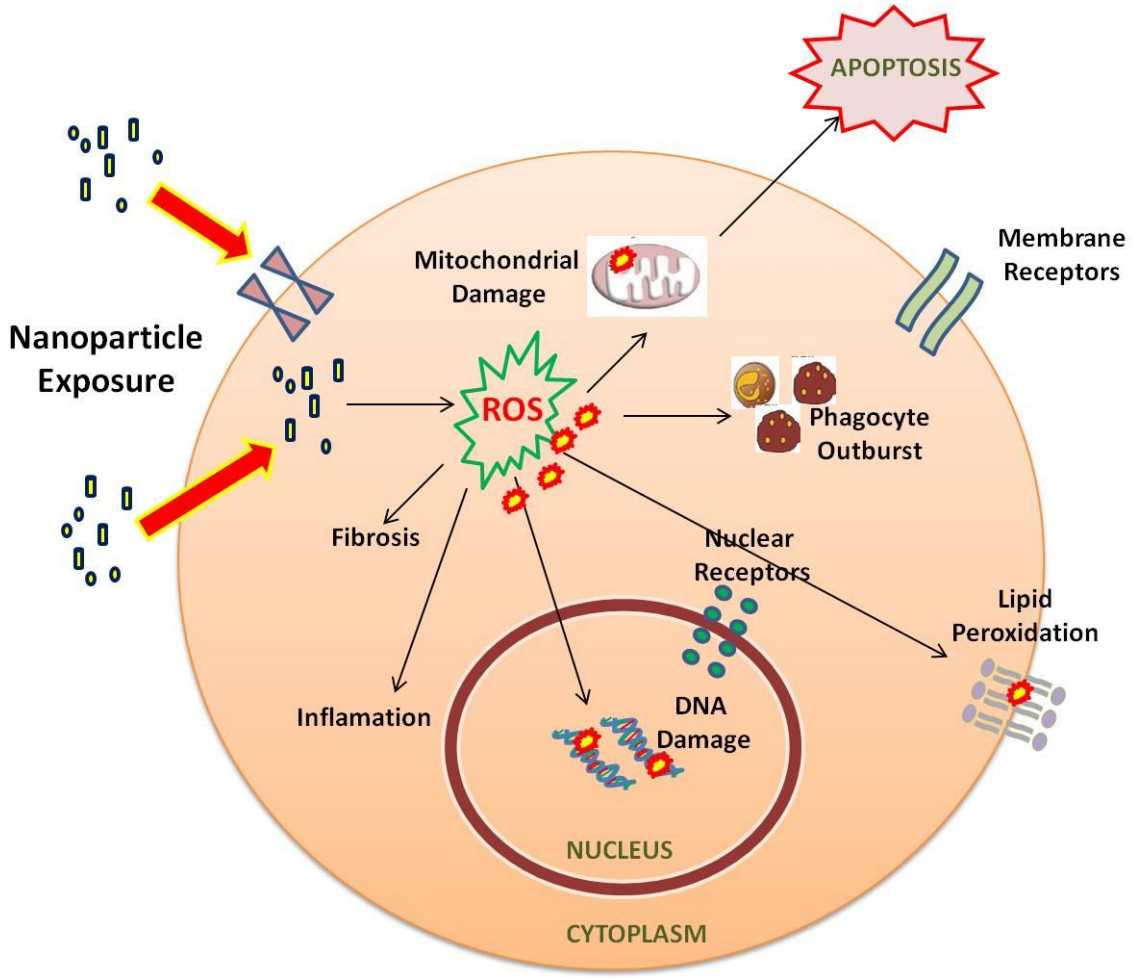

Figure 1: Nanoparticle mediated cellular responses: NPsmediated ROSgeneration is capable of inducing oxidative DNA damage, strand breaks, protein denaturation, and lipid peroxidation. ROS alsoresults in mitochondrial membrane damage that leads too cellular death by Apoptosis.

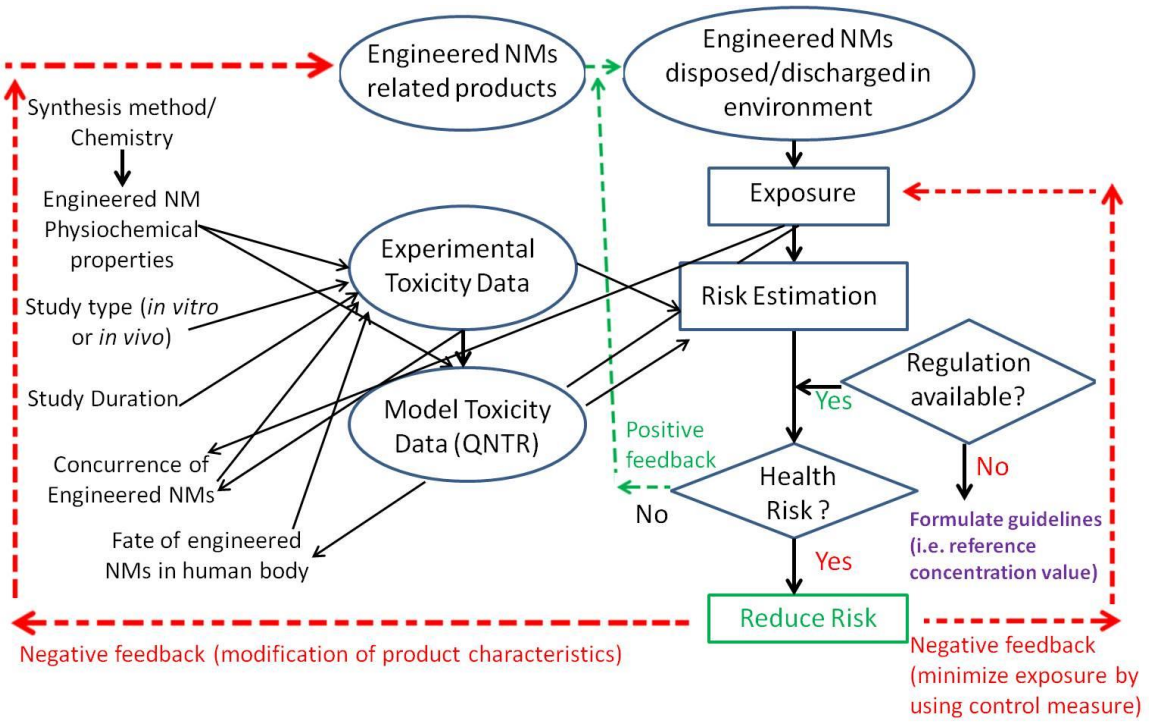

Figure 2: Interlink of different factors for determining environmental and health risks due to Engineered NM exposure -- a schematic representation (Courtesy:(Kumar et al. 2014)).

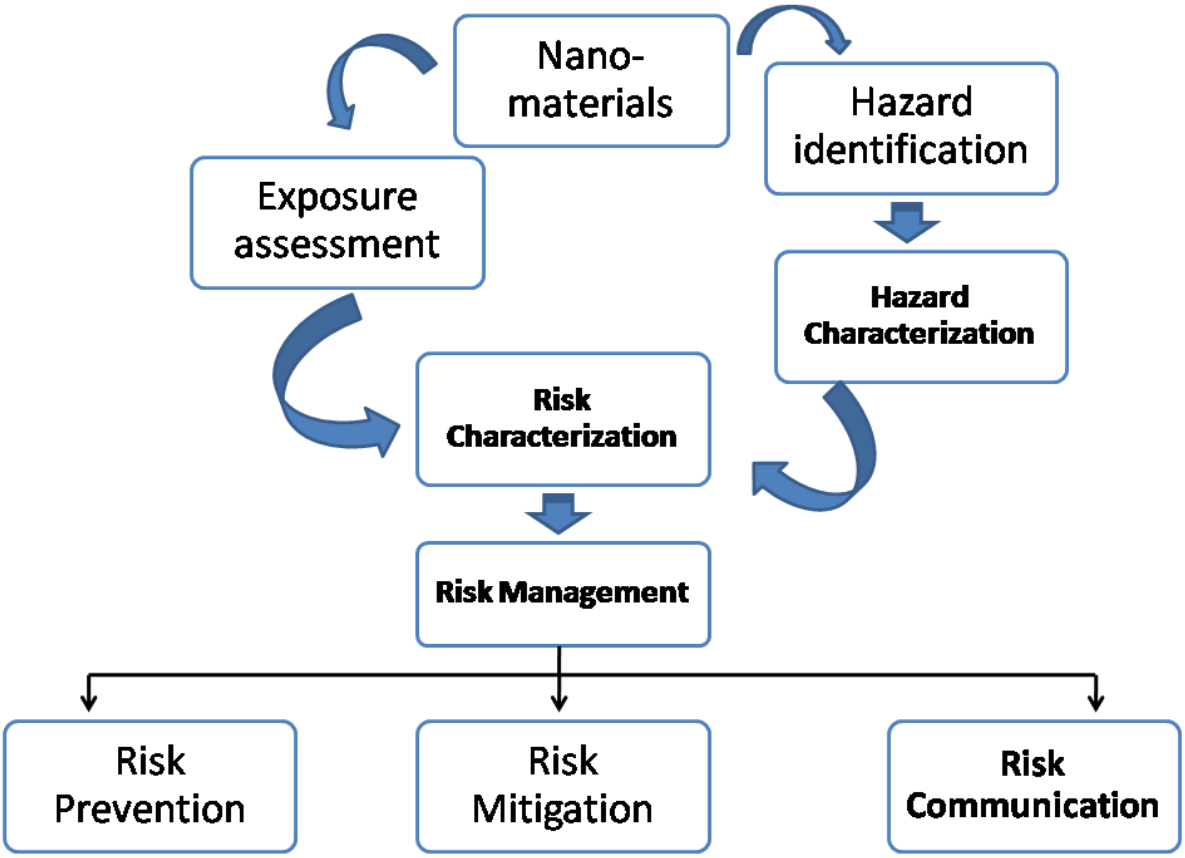

Figure 3: The toxicological aspect of nanomaterials on humans, animals, environment and whole ecosystem. Diagrammatic representation of overview of nano-toxicological analysis (*Courtesy:* **Dasgupta et al. 2016**).