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Nanonutraceuticals
Are They Safe?

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17.1 Introduction

New technologies offer significant benefits to humans, but they also possess multiple risks to human and environmental health. Nanotechnology has seen exponential growth in the last decade due to its unique physicochemical properties (Kingsley et al. 2013). However, the risk associated with this emerging technology is also due to the small size and large surface area of nanoparticles (NPs), which allow easy dispersion, but might cross anatomical barriers and show potential toxicity. NPs could enter the food chain via 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, biodistribution, entry in the food chain, and so on need a thorough investigation before NPs 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 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, Rashmi, and Rao 2010; Kalpana, Anshul, and Rao 2013). The small size and successive larger surface area of NPs are some extremely valuable and precise properties, but this also makes them biologically more active leading to unpredicted and unexpected consequences on interaction with biological structures. The smaller size also conveys a dissimilar biokinetic behavior and capability to reach extra distal sections of the body (Oberdörster, Oberdörster, and Oberdörster 2005). The work-related introduction of NPs will also amplify with the growing production and use of nanomaterials (NMs) socially.

Environmental contamination is hitherto an additional concern, especially the probable undesirable effects of engineered nanomaterials (ENMs) upon the human and the environment healthiness. Government, regulatory authorities, and scientific authorities all over the world are realizing the importance of nanomaterial risk assessment. **Figure 17.1** depicts the interlinked different factors for determining environmental and health risks due to ENM exposure.

A systematic knowledge of the mechanism of NPs flowing in and out cells could also lead to a enhanced understanding of NP toxicity including enhancement in their biomedical 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 titled "Opinion on Safety of Nanomaterials in Cosmetic Products." The report raised concerns about huge data gaps, the 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; Dhawan, Sharma, and Parmar 2009; XpertArena 2015).

17.1.1 Exposure to nanomaterials

Every day we are exposed to a number of nanomaterials, whether anthropogenic or natural. The manufacturing units of nanomaterials also pose a threat

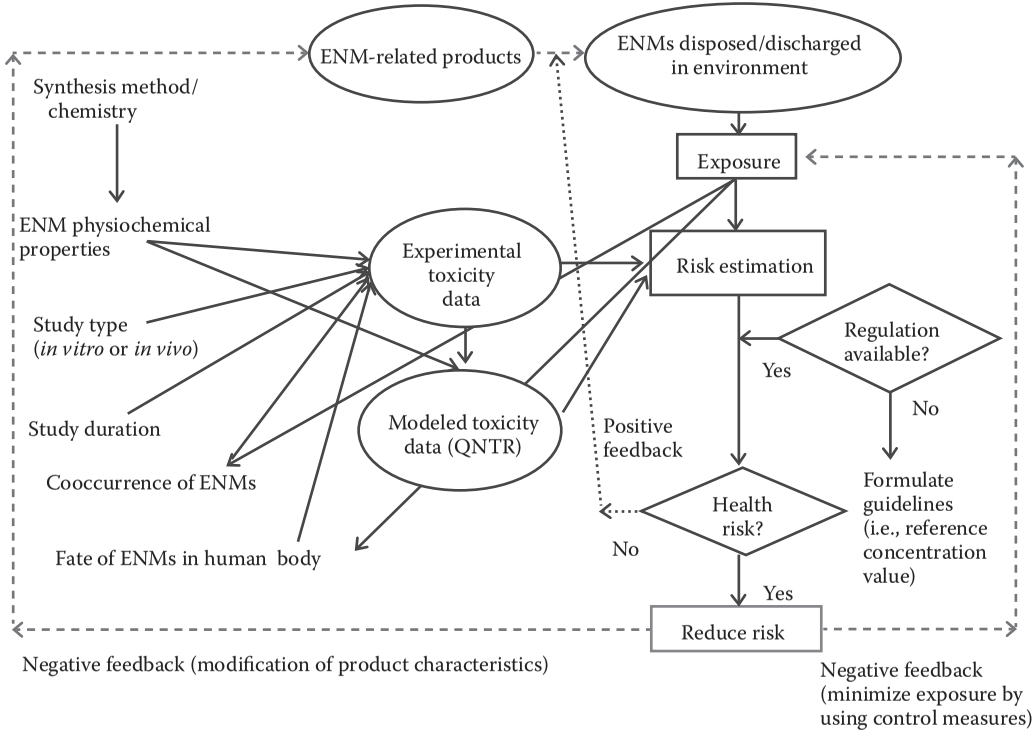

Figure 17.1 *Interlink of different factors for determining environmental and health risks due to ENM exposure, a schematic representation. (Courtesy of Kumar, Arun, Prashant Kumar, Ananthitha Anandan, Teresa F. Fernandes, Godwin A. Ayoko, and George Biskos, 2014, "Engineered Nanomaterials: Knowledge Gaps in Fate, Exposure, Toxicity, and Future Directions," Journal of Nanomaterials, article 130198.)*

to exposure to humans or the 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. The washing off of these consumer products results in the entry of nanomaterials into the environment (Kumar and Dhawan 2013a,b; Mihranyan, Ferraz, and Stromme 2012).

Inhalation is one of the most common routes of exposure to NPs (Bakand, Hayes, and Dechsakulthorn 2012). The large-scale production of powder during NM synthesis, processing, and/or packaging also possess a threat to the workers engaged in these industries. Lack of regulatory checks on the manufacturing units also adds to the chance of leaking of NPs to the environment. These airborne NPs pose a lethal effect to the health through the respiratory system (Kim et al. 2009; Jasmine et al. 2010). Any overseas particle inflowing toward the 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 other organs such as the liver and spleen (Dhawan et al. 2011). These particles are then either eliminated or retained within the body and again translocated to other organs.

Dermal exposure is another exposure route for NM entry. The NM barrier is still to be completely explored. Transappendageal, intercellular, and transcellular can be possible routes for NMs (Wu et al. 2013; Yan and Chen 2013). NMs soluble in lipid may move through lipid-rich membranes among skin cells inside the intercellular routes, whereas with the transcellular route the substance penetrates the skin cells. Hair follicles and sweat glands spread all around the skin in various densities may become the means for NM entry for the transappendageal route (Albanese and Chan 2011; Crosera et al. 2009; Love et al. 2012).

Direct ingestion of NMs occurs when they are utilized in drug delivery, food, food packaging, and cosmetics. Apart from them, 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. Swallowed NPs possibly will be translocated via the lumen of the intestinal tract into several organs (Pietroiusti, Campagnolo, and Fadeel 2013). A study by Böckmann et al. (2000) reported translocation of TiO_2 NPs to different organs through the gastrointestinal (GI) tract via the blood. The extent of uptake is also dependent upon the size and shape of the NPs. Triangular-shaped NPs are found to be more toxic than spherical nanoparticles (Chan et al. 2008; Dasgupta et al. 2015c; Huang et al. 2007). 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 was translocated from the Pyer's patches into the mesenteric lymph and then to systemic organs (Jani et al. 1990).

17.1.2 Risk assessment

Recent years have witnessed use of nanomaterials 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 the 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 nanomaterials. In June 2003, UK officials asked The Royal Academy of Engineering and The Royal Society to look into the benefits, safety, and health issues arising from usage of nanomaterials. The Royal Society published its report in 2004 titled "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, Kumar, and Dhawan 2012; Tervonen et al. 2009).

The United States Environmental Protection Agency (USEPA) is also actively working in potential usage of nanomaterials and the risks associated with it. It also stresses development of NMs with a practical approach. In its document EPA 100/B-07/001 (Nanotechnology White Paper) published in 2007, it stated "as the use of nanomaterials 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 nanomaterials 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 and issues to be considered in conducting risk assessment on nanomaterials (Sharma, Kumar, and Dhawan 2012). The 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 nanoparticle risk assessment, and inadequate information

regarding nanoparticles absorption and uptake in both normal and diseased skins. Guidance documents on harmless management of NMs are also being outlined by researchers. Nongovernmental 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 nanoparticles 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; Ranjan and Dasgupta 2013; XpertArena 2015).

17.1.3 Usage of nanomaterials in food

Nanotechnology is also used in the food sector as nanosensors, tracking devices, targeted delivery of required components, food safety, new product developments, precision processing, and smart packaging (McClements et al. 2009; Huang, Yu, and Ru 2010; Yu and Huang 2013). Nanostructured materials exhibit unique physicochemical properties that open windows of opportunity for the creation of new, high-performance materials that would have a critical impact on food manufacturing, packaging, and storage. Currently, the application of nanotechnology in the 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, nanocoating that protects tomatoes from humidity and oxygen, bread containing nanocapsules of omega-3 fatty acids, and juice containing vitamin A encapsulated in starch, and use of nanocans for packaging and transportation of liquid beverages because of their lightweight are gaining acceptance 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 (i.e., food additives, nutraceuticals), but the long-term focus can be brought upon controlled release of nanoencapsulated food ingredients or nutrients (Dasgupta et al. 2015a; McClements 2015). Nanotechnology can also improve the water dispersibility, thermal stability, and oral bioavailability of the functional compounds of food (McClements et al. 2009). Various applications of NPs in the food industries are globally focused on (a) sensory improvements (flavor/color enhancement, texture modification), (b) increased absorption and targeted delivery of nutrients and bioactive compounds, (c) stabilization of active ingredients such as nutraceuticals in food structures, (d) packaging and product innovation to increase shelf life, (e) sensors to assess the safety of food, and (f) as antimicrobial agents against foodborne pathogenic bacteria. The stability of nanomaterials in food is dependent on a range of storage conditions (low and high 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 (Monica and van Calster 2010; 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, coloring strength, processability, and stability during shelf life, which help to increase physiochemistry of food.

17.2 Characterization of nanomaterials for toxicological evaluation

17.2.1 Characterization of nanomaterials in biological matrices

The behavior of NPs in the biological system greatly depends upon its surface characteristics. On the other hand, NMs require widespread characterization, unlike chemical compounds, where the characterization is typically limited to chemical composition as well as purity determination. This is because the precise properties of NPs as well as their correlation with its biological activity are inadequately understood. As a result, additional widespread and complete characterization, with surface area, surface chemistry, size distribution, shape, porosity, agglomeration state, crystallinity, surface charge, solubility, and so on is strongly suggested for NMs characterizations in order to determine the accurate correlation among their physicochemical properties and the biological effects elicited by them. Of 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 gives 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 that are specified by the manufacturer occasionally vary from those established by the researcher (Sayes, Reed, and Warheit 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 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.

Many techniques exist 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). However, these methods obtain conflicting size distributions and average sizes. This is apparently not unexpected considering the diverse basic principles behind the techniques. Additionally, deviations in sample preparation scheme and apparatus operational procedures also add to measurement dissimilarities. However, this might lead to misunderstanding regarding the concrete size and size distribution of NP. Also operators are often not experienced in the principles and practical details of the measurement techniques.

The U.S. National Institute of Standards and Technology (NIST) has created 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. The detailed measurement protocols including the data results 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 as interlaboratory comparisons.

When it comes to the NP toxicity, NP surface area is an important factor, because the interaction between NPs and biological systems 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 of a liquid nitrogen monolayer on the particles' surfaces and then estimating the quantity of nitrogen unconstrained upon vaporizing that layer. Therefore, the BET surface symbolizes the surface area that 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 are already available for the protocols. Although, the advantage of this technique is that it affords two parameters at the same time (surface area and size), its downside is that it presupposes average-sized spheres containing a monodisperse system, so it does not give an 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 straightly measures size distribution, size, and morphology for materials. On the other hand, it is time taking and needs a enough number of materials which contain 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 unable to determine the particles' properties in the dispersion form, which is used for investigational revelation (Powers et al. 2007).

An atomic force microscope (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. An AFM offers visualization in 3D 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 the 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 recommends a wider range, of NPs from 1 nm to 8 μm, and can to calculate with a single scan (Scalf and West 2006). Furthermore, it involves less laboratory space than TEM/SEM and is much easier to use.

Dynamic light scattering (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. This can be attributed to the fact that DLS measures Brownian motion and the subsequent size distribution of an ensemble of particles in solution and yields 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). With DLS measurements, there is a tendency of NPs to aggregate in the aqueous form, so it provides the sizes of cluster NPs rather than a single NP. It provides an intensity weighted average hydrodynamic diameter of a group of NPs, so any sample polydispersity will skew the average diameter in the direction of larger NP sizes (Dhawan, Sharma, and Parmar 2009). This method gives additional afford 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 provide 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 medium and time. Murdock et al. (2008) demonstrated the effectiveness of DLS by analyzing the reliance of the in vitro toxicity estimation of NPs on the dispersion state, the medium of exposure, the serum presence, the time gap among exposure, and sample preparation. It is an assembly method where the amount of a compilation of NPs is used to estimate the NP size distribution.

Recent studies based on the Brownian motion of NPs are called 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. It circumvents any intensity bias near large NPs that could result in a small number of large agglomerates/particles masking the existence of a large number of NPs, as noticed with other light-scattering techniques (e.g., DLS). 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 weight as shape, size, and agglomeration, however, the role of the NPs' surface properties in their toxicity and how these properties are modified

throughout exposure under the influence of diverse environments are sought, 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 tunneling microscopy are a few surface analytical technique to give information regarding elemental composition, topography, molecular and chemical state, and 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 coworkers (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 milligrams), but they must be representative of the whole sample. Various ways of performing reliable powder sampling and some general error connected with sample preparation have previously been discussed in detail by Powers et al. (2007). The NPs properties in liquid suspensions be liable to alter the surrounding environment and with time. NPs physical properties prior to exposure may change once the NPs 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 multitechnique analysis so as to get a broader perception and more dependable photographs of the NPs' characteristics. Cooperation among many laboratories that possess expertise in their relevant methods need to be encouraged. A sufficient number of NPs should be calculated to get statistical significance.

17.2.2 Measurement of nanomaterials in food and other biological matrices

Nanomaterials vary in their size and shape, and also may undergo modifications during processing and manufacturing. Once ingested, nanomaterials also interact with different biological materials. Although some characterization techniques are present, there is no single method applicable to all nanomaterials to predict their safety for consumption. The different physical and chemical properties also make it difficult to develop a single characterization method. Thus, a combination of techniques can be employed to predict potential benefits or risks (Kettiger et al. 2013; Kunzmann et al. 2011; Magnuson, Jonaitis, and Card 2011). From the current techniques available, it is now possible to identify if nanoparticles are present or not in biological matrices. Inorganic nanomaterials, primarily silver, gold, and silica nanoparticles, 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). To determine the best combination of methods, one has to list the objective for the sought after characterization. One can simply find out if nanomaterials are present or the changes incurred upon the biological matrices after interaction with nanomaterials. Detection methods can also be done to analyze the commercially available products to find if the nanomaterials added have changed its properties similar to its bulk counterpart during 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, nanomaterials' chemical composition also affects its properties and the extent of translocation of these nanomaterials from the gastrointestinal tract to different organs via blood. Inorganic nanomaterials if present in their ionic form are reported to be more toxic than their 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 nanomaterials, however, the same cannot be said for organic nanomaterials comprised of proteins, lipids, polymers, and polysaccharides that resemble biological materials. Elemental nanomaterials are easier to detect and quantify than organic. To detect any nanomaterial from a given biological sample such as food matrix or intestinal cells, the sample must be digested to release the nanomaterials. Once extracted, some of the aforementioned techniques may be utilized to determine the presence of nanomaterials. Enzyme-linked immunosorbent assay (ELISA) kits for antibody-based detection and flow cytometry are the rapid screening techniques that may be useful for detection of organic nanomaterials (Dehalu et al. 2012). Mixed nanomaterials (e.g., inorganic core with organic coating) need a set of paired techniques, mainly electron microscope combined with sample chemistry-based methodology, for example, x-ray photoelectron spectroscopy (XPS), scanning probe microscopy (SPM), scanning tunneling microscopy (STM), AFM, low energy ion scattering (LEIS) technique, and secondary electron mass spectroscopy (SIMS) (Baer et al. 2010; Kettiger et al. 2013; Magnuson, Jonaitis, and Card 2011).

Many companies considering these advanced instrumentations too costly and time-consuming in comparison to other techniques, such as high-performance liquid chromatography (HPLC) and dynamic light scattering (DLS). Due to the several challenges, attaining informative data from complex materials methods have not been well validated for characterizations of organic and inorganic nanomaterials in food and drugs (Corredor et al. 2015; Wise and Brasuel 2011). For example, chemical imaging techniques and electron microscopy techniques only provide useful nanomaterial image data when the samples have large changes in disparity (chemical as well as optical respectively or both) between the nanomaterials and the surrounding matrices. This creates the challenge to locate nanomaterials such as carbon nanotubes within cells and tissues rich in carbon. Moreover, this complicates sample preparation

methods because of labile behavior of nanomaterials, which can ultimately result in image data that cannot be distinguished between concepts, for example, engineered nanomaterial migration versus agglomeration. Labeling organic nanomaterials via fluorescent tags or radiolabels may be the potential troubleshoots to some of these issues. However, such modifications may change the chemical or physical characteristics of organic engineered nanomaterials to make them poor models of their unlabeled versions. It is challenging to develop reliable methods for imaging nanomaterials in food matrices and alimentary tract cells/tissue than those based on present detection technologies. Researchers need to develop new analytical approaches for organic nanomaterial sampling, detection, and quantification, as well as imaging of both inorganic and organic nanomaterials. Additionally, it is needed to assess the hazard of nanomaterials in food, drug, food/drug contact materials, and the alimentary canal (Alger et al. 2014; Ostrowski et al. 2015).

17.2.3 Problems associated with measurement and characterization of nanomaterials

Although there are a number of characterization techniques available, some shortcomings complicate the development of methods used to identify NMs used in food and other biological matrices. The first thing that needs to be identified is whether the NMs are naturally present 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 nanoparticles even before the processing steps have been applied (Dasgupta et al. 2015b). 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 nontoxic 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 initially pure form, tend to have similar characteristics at the manufacturing unit, which are easier to identify, characterize, and quantify. Once these NMs are exposed to food matrices, some changes are conferred upon to both the 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 the gastrointestinal 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 a specific combination of characterization techniques 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 the purpose of the NMs used in food. NMs, whether natural or manufactured, have been used for a variety of purposes including increasing shelf life, appearance, rheology, stability, and texture, or for organoleptic characteristics. Thus, a variety of analytical approaches is required to get desired results (Hwang et al. 2012; Yada et al. 2014).

Prior to characterization techniques, the sample (food matrices or biological material) has to undergo the extraction process to "release" the NMs. Incorporation of NMs to food matrices also alter their reactivity. Thus, a single extraction method is not applicable for all types of food products. The pretreatment 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, Peralta-Videa, and Gardea-Torresdey 2013).

17.3 Toxic effects of nanomaterials

17.3.1 Genotoxic potential

As the NMs are absorbed through the gastrointestinal tract, they interact with various types of cells, proteins, and even DNA. Due to their small size and high reactivity, the probability of their internalization into the cells and interaction with cellular organelles and macromolecules (DNA, RNA, and proteins) are very high. These interactions can alter the genetic material and induce mutation, or can 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 reactive oxygen species (ROS) generation and release of toxic ions from NMs (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 NM interaction with cytoplasmic/nuclear proteins, disturbance of cell cycle, oxidative stress, ROS generation, or binding with mitotic spindle or its components. Interruption of antioxidant defense by NMs also induces genotoxicity (Kumar et al. 2015; Dhawan and Sharma 2010; Kansara et al. 2015; Shukla et al. 2013b).

Using a computational approach, it was observed that during DNA replication, carbon nanotubes can bind to sister DNA strand and get integrated into the DNA duplex. This also suggests that carbon nanotubes can hinder the DNA replication process. Apart from carbon nanotubes, other NMs are also reported

to show strong interaction with the DNA and DNA bases in different organisms (An et al. 2010; Jin et al. 2012). Another in silico study showed disturbance of the DNA mismatch repair pathway by C60 fullerene by possible interaction with PMS2, RFC3, and PCNA proteins. Another 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 the 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) showed DNA repair activity in A549 cells was impaired by TiO_2 nanoparticles. 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 that regulate biological functionalities of many systems such as the mitotic spindle apparatus, DNA replication, centrioles, transcription and repair, and associated proteins. The interaction studies are based on computational and in vitro studies. Signaling pathways can be activated by low concentrations of ROS. On the other hand, at higher concentration it induces many damages, including cell membrane, mitochondria, and other macromolecules damage, and lipid peroxidation. The major source of the oxygen-free radicals and a major target of ROS-induced oxidative stress and damage is the mitochondria. Various proapoptotic factors are released by mitochondria under stress conditions due to the depolarization of the intermembrane potential and an increased permeabilization of the outer membrane (Kumar et al. 2011a; Cadenas and Davies 2000; 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), which plays a major role in carcinogenesis and mutagenesis. 8-oxoG can be considered an indicator for DNA damage because of oxidative stress after NM exposure, which has been analyzed by FPG-modified comet assay (Asare et al. 2012; Kim et al. 2011; Magdolenova et al. 2014). It should be noted that a level 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 NMs induced genotoxicity can be inhibited by pretreatment with the free radical scavenger *N*-acetyl-L-cysteine (NAC) (Guo et al. 2011; Sharma, Anderson, and Dhawan 2012). This ultimately helps to explain the mechanism of ROS-induced cellular perturbation along with apoptosis and DNA damage.

17.3.2 Carcinogenic potential

DNA damage and mutations are induced by NMs; this fact has been established by several in vitro and in vivo experimentations and the well-known

association between genotoxicity and cancer has been established. Therefore, these analyses provide very useful information about the carcinogenicity of NMs, for example, the 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, Morris, and Cronin 2005). The antioxidant defense mechanism of the cells is increased by excessive generation of ROS because of oxidation of biomolecules. It has been well recognized that oxygen-derived species have a main role in causing cell injury or death. A large number of degenerative changes lead to tissue degradation because of the involvement of ROS, which ultimately causes carcinogenesis, aging, and other diseases (Luo et al. 2011). Additionally, it also affects the immune system, which further leads to an increased microbial load resulting in cell and tissue damage. The cancer-causing types of genetic changes are 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 tumors elevation of 8-OHdG has been reported, which strongly associates such damage in the etiology of cancer. Several cell-line-based studies suggest the carcinogenic potential of NMs because of their capability to induce the level of 8-OHdG in cells.

The initiator of carcinogenesis—oxidative stress—can be induced by NMs, which can induce inflammatory responses. The presence of electrons on the NM boundary is the main factor for their high reactivity. NMs react with proteins and enzymes faster, adsorb endogenous substances, and trigger cytokine release, which is responsible to mediate 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). Likewise, exposure of carbon-based NMs—mainly carbon nanotubes—causes platelet aggregation, aortic DNA damage, and enhanced vascular thrombosis through inflammatory events, which results in adverse cardiovascular effects.

17.4 Toxicity evaluation: Methods and techniques

Various models such as in silico, microbial system, and cell culture in vitro and in vivo models can be used to assess the genotoxicity of NMs. The Ames test has been extensively accepted to assess the genotoxicity of a variety of NMs (Kumar et al. 2011b; Maenosono, Yoshida, and Saita 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 that are mutated at the histidine locus. As such, they do not synthesize histidine and thus die when plated on an agar medium lacking histidine (Ames, McCann, and Yamasaki 1975; Mortelmans and Zeiger 2000). However, compound/NMs will enable the bacterium to synthesize histidine due to reversal of mutation in the histidine gene. The bacteria form colonies in a minimal histidine medium. A bacterial cell wall can be modified with deep rough (rfa) mutation, which eliminates the polysaccharide side chains of lipopolysaccharides, to make the bacteria more permeable. As the bacterial cell wall is rigid and semipermeable, it allows only 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 assay hypoxanthine phosphoribosyltransferase (HPRT), comet assay, phosphatidylinositol glycan, thymidine kinase (TK), 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 assess of the genotoxicity of a substance (Finette, Kendall, and Vacek 2002). The cell lines used have one functional copy of the HPRT gene located on the X chromosome. This gene is involved in phosphoribosylation of hypoxanthine and guanine. A toxic analogue 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 the HPRT enzyme leading to cell death. However, if the compound or NMs induce 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 NMs. The micronucleus is a chromatin-containing structure formed from the lagging chromosomes or their fragments during the anaphase stage of the 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) that 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 the comet assay. It is a rapid, simple, sensitive, and frequently used technique. It is used to detect oxidative DNA damage, abasic 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, Johnston, and Schins 2009). Single cells 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 (e.g., 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) interference was shown, which was found to induce additional DNA damage (Karlsson 2010). Also, it has been found that NMs and ions released due to 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, Karlsson, and Möller 2012). The inhibition can be justified by the fact that ions are getting bounded 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 the nucleosome core histone H2A family. The phosphorylation of this protein at serine-139 is mediated by ataxia telangiectasia mutated (ATM), 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 break site. The alteration in the expression profile of γ-H2AX induced by ENPs has been detected by different techniques such as immunohistochemistry, flow cytometry, and Western blot (Ismail, Wadhra, and Hammarsten 2007; Lewis et al. 2010).

17.5 Conclusion

Many more advancements are required to develop nanofood technology into a fully functional branch of modern science. Some of the future aspects that need special mention are characterization and standardization of the various

research parameters (which may vary from instrument to instrument and also due to differences in methodology adopted), methodical characterization and standardization of analytical instruments, and development of standard protocols to identify and characterize biological and food matrices. Further, efforts can be taken by leaders in the field to develop a proper method to validate and quantify various data produced by different groups of workers worldwide. Detection and identification of nanomaterials in working samples, especially in biological samples, is at times very challenging.

Advancements in food processing, packaging, proper delivery of nutraceuticals, and quality control with nanosensors have benefited the farming community. In the near future, many such products can be developed for efficacy. Development of an integrated nanomaterial–microbial system to be used as a water purifier; food-grade nanoemulsion to be used in fruit juice and other drinks; nanomaterial conjugated with activated charcoal to be used in agricultural products' processing and storage so as to enhance their antimicrobial activity; plant extracts to be conjugated with nanofood packaging for enhanced properties; and nutraceutical delivery efficiency improvement using nanotechnology are some of the future directions in the field. Though several research groups worldwide are working on applications of nano-agrotechnology, there is only very meager understanding on the toxicological aspects of nanoparticles and the mechanism of nanotoxicology among the scientific community (Maddinedi et al. 2015).

Though the era of nanotechnology is in the naive stage, it is fast evolving and has become one of the fastest growing branches of modern science. At present, every field in science is in one way or the other related to nanotechnology. From farming practices to marketing and trading, nanotechnology has brought about a revolution. Food and agriculture are the two major sectors that have infused confidence among scientists about the credits and credibility of nanotechnology. Synthesis and large-scale application of nanoparticles in the agriculture sector led to a revolutionary change resulting in high productivity in some of the major areas. Agricultural water quality management (WQM), agro-product processing, storage and distribution, quality control with nanosensors, and so forth have brought about huge profit to farmers.

Additionally, more research is needed for the application of nanotechnology in aquatic and terrestrial systems as well as their interaction with organisms and biomolecules. It is needed to enhance the knowledge and awareness of nanotechnology applications in both agriculture and farming systems, such as fertilizers, enhanced nutrition, WQM, and biosensors, for farmers as well as industrial personnel and researchers. Nano-education should also connect schools, colleges, research centers, small-scale industries, and consumers to understand the potential benefits as well as risk and safety aspects of nanotechnology.

Schemes should be formulated and methods have to be developed to detect the presence of nanomaterials using proper instruments. Furthermore, a

proper correlation between size and physicobiological properties like toxicology, cell–cell interaction, and the effect of bioactive compounds in the presence of nanocompounds are altogether not yet explored. Unlike proteins or other biological samples that can be detected and quantified, presence of nanomaterials cannot be quantified in samples. Unless the nanomaterials are properly detected and quantified, it is futile to design further systems. There is a severe lack of scientific data among research communities for different regulatory agencies (like the U.S. Food and Drug Administration or World Health Organization) to assess and provide risk management guidelines. Advances in these directions will be gratifying and can indeed contribute to the emergence of nanotechnology as a fast expanding branch of science.

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