Chapter

Challenges for Assessing Toxicity of Nanomaterials

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Abstract

On the development of nano-world, nanotechnology provides enormous opportunities in daily routine products and further future sustainable innovations. The nanotechnology extends its benefits to various fields such as engineering, medical, biological, environmental, and communication. However, the exponential growth of nanomaterials production would lead to severe complications related to their hazardous effects to the human health and environment. Moreover, negative impact of nanomaterials toxicity on human health is one of the significant issues on exhausting nano-products. The most vulnerable situation is associated with the use of nanomaterials in the biomedical application. The several efforts have been ongoing to study the nanotoxicity and its interaction with the biomolecules. Nevertheless, it is hard to assess and validate the nanotoxicity in a biological system. This chapter aims to study the challenges in determining the toxicity of nanomaterials. The toxicity assessment and hurdles in determining the impact on biological systems are epoch making. *In-vitro*, *in-vivo*, and *in-silico* studies are summarized in this chapter in assessing the toxicity of engineered nanomaterials. The different approaches of toxicity assessment have their difficulties faced by researchers while characterizing nanomaterials in powder form, solution-based, and interacting with biological systems. The assessment tools and characterization techniques play a vital role in overcoming the challenges, while the cytotoxic assays involve nanoparticle shape, morphology, and size consideration.

Keywords: nanotechnology, nanoparticles, characterization, *in-vitro*, *in-vivo*, *in-silico*

1. Introduction

In today's high-tech world, nanotechnology has become so much popular in various fields due to its unique and beneficial physicochemical properties [1]. Some of the essential applications in multiple areas have been mentioned in **Table 1**. Bringing the materials to nanoscale level helps in improving mechanical, optical and electrical properties. It can be explained due to the increase in surface area to volume ratio and hence, surface-related properties become more significant.

The small size and higher specific surface area of NMs furnishes the distinctive properties and leads to unpredicted biological response on interaction with biological system. Further, they also impart different biokinetic behavior and capabilities to reach farther in body as compared with their larger counterparts.

Applications	Usage	Ref.
Nanomedicine	Fluorescence and multiphoton bioimaging, *in vitro* diagnostics, *in vivo* fluorescence imaging, drug delivery, photodynamic therapy, Photothermal-controlled drug delivery and cancer treatment, drug release, bioimaging, Tissue engineering, gene therapy, regenerative medicine, MRI, magnetically guided control drug delivery, magnetic biosensing, Drug release and gene delivery, gene material and vaccines	[2]
Health sector	Therapeutic targets in chemotherapy; bio-nanosensors; nanocoatings; nanocarrier for vaccination; antimicrobial activities; nanophotothermolysis for cancer, nanofilter, cosmetic products,	[3–6]
Food and agriculture	Nanofertilizers, nanofungicides, nanopesticides, engineered nanomaterials, CNT (carbon nanotube), nanoporous membrane, food-based nanodelivery vehicles, food storage and packaging, functional foods, bio-actives, nutraceutical systems, and pharma foods	[7–11]
Energy and environment	Wastewater treatment, adsorption and degradation of organic/inorganic pollutant, nanofilters/membranes, Solar energy, energy storage, H_2 generation, Li-ion battery	[12–20]
Defense and security	Smart materials, fuel additives, modern weapon, nanocoatings, nanocomposites, night vision camera, sensors and electronics, and energy devices, robotics	[21–24]
Automobile	Nanomaterials in paints, nanocoatings, catalyst as additives, nano-based lubricants, fuel cells, composite fillers, smart lights	[25–27]
Building	Pigment in Interior and exterior paints, as a thin film on glass windows, photocatalyst, adsorbent, as a membrane, hydrophilicity, climate control, sensors, Rheological behavior under uniaxial extensional flow, improved mechanical properties, fire retardant and insulation, cement composite	[28]
Electronics device	LED, OLED, nanotransitor, nano-based memory device, opto-magnetic, spintronics, electrochromic device, nanogenerator	[29–31]
Textiles	Smart fibers, stain repellence, wrinkle-freeness, nanocoatings, high absorbency, softness and breathability, military applications	[32, 33]
Sports	Nanofibers, ball coatings, CNT based sports items	[34, 35]

Table 1.
List of numerous applications of nanomaterials in different area.

With the increasing use and production of nanomaterials (NMs), occupational exposure is also growing. Other concern is related to environment and ecosystem disturbance. Some of these apprehensions have forced scientist to investigate and understand the potential adverse effects of engineered nanomaterials on health and environment and also, explore the challenges to assess the toxicity of these materials. Several reports on toxicity assessment of NMs published in the last few decades. However, still it is a challenging task to investigate the interactions of nanoparticles (NPs) with biological systems. One of the probable reasons could be due to experimental methods and precise characterization involving toxicological assessment of NMs. Although, human health is at considerable risk because of toxicity of these nanotechnology-based goods on exposure/intake by several routes (**Figure 1** and **Table 2**) [36].

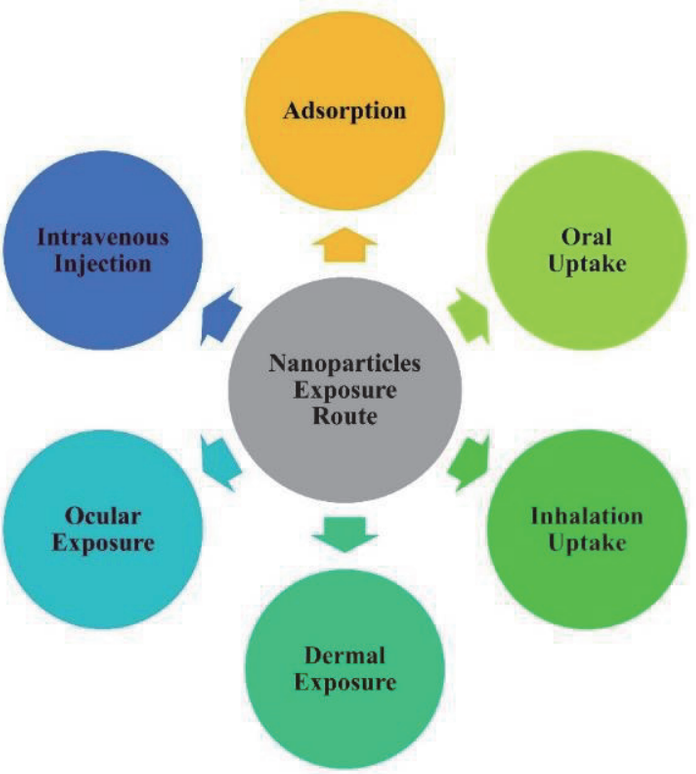

Figure 1.
Exposure pathways of nanoparticles.

Nanomaterials	Possible risks
Carbon, silver and gold NPs	Affect the central nervous system, respiratory toxicity, liver toxicity
Carbon NPs	Pulmonary inflammation, granulomas, and fibrosis, inhibition of DNA enzymes, enhanced cytotoxicity, pulmonary toxicity
Cd-based compounds	Nephrotoxic potential, cell and DNA damage, lungs and liver toxicity, fetus malformation, hampered growth, enhanced cytotoxicity
CuO NPs	Suppress immune system, cell and DNA damage, toxic to aquatic organisms
Ceria NPs	Reactive Oxygen Species (ROS) production, decreased lifespan, cell membrane and DNA damage, lipid peroxidation
TiO_2 NPs	Genotoxicity, metabolic change, neurotoxicity, skin penetration, cell damage, ROS production, reproductive toxicity
ZnO NPs	Hepatic oxidative stress, severe liver damage, reproductive toxicity on earthworms
QDs (Quantum Dots)	Lung infection and inflammation, fetus malformation, hampered growth, sperm count and quality decreases, cell damage
SiO_2 NPs	Chronic obstructive pulmonary disease, tuberculosis. Lipid peroxidation and membrane damage, mitochondrial dysfunction, lung cancer, cell death (necrosis)
Nano-MOFs	Reproductive and respiratory toxicity, immunotoxicity, neurotoxicity, carcinogenicity

Table 2.
List of nanoparticles causes possible toxicity to the human body [2].

2. Challenges in characterization

To study the toxicity of any chemical substances, characterization of materials plays a significant role. There are several techniques available these days which can be used to characterize nanomaterials in powder form, film as well as in solution and further its interaction with biomolecules can be studied (**Figure 2**). Although, it becomes much more imperative and extensive in case of nanomaterials due to the different shapes and sizes with variable surface area, charge and chemistry, crystallinity, porosity, agglomeration, solubility, etc., (**Figure 3**). Further, the nanomaterials generated from experiments must ensure reproducibility of

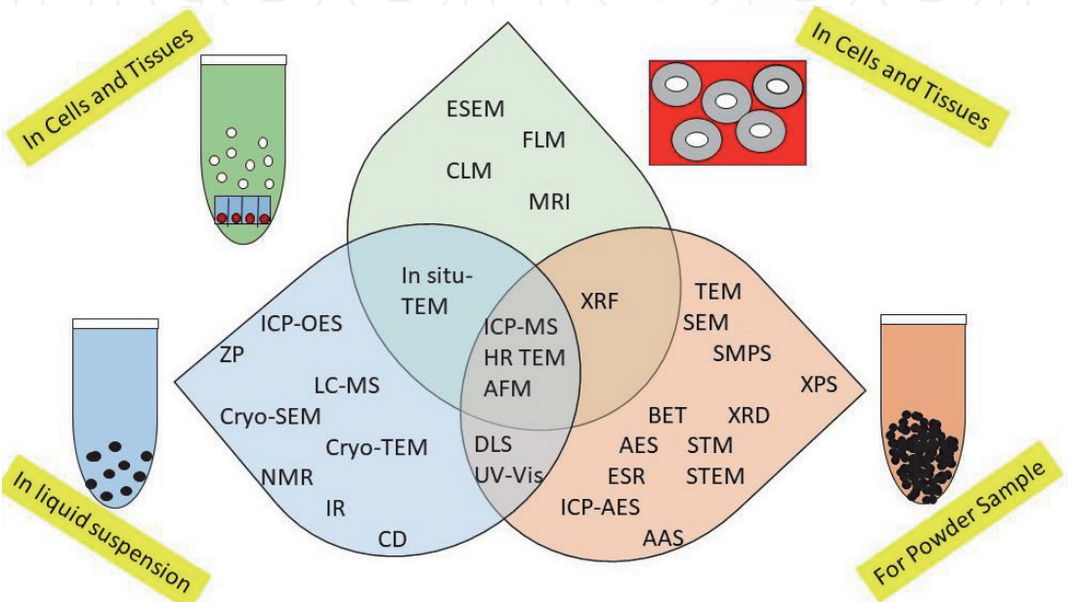

Figure 2.
Characterization of nanoparticles in different media [37].

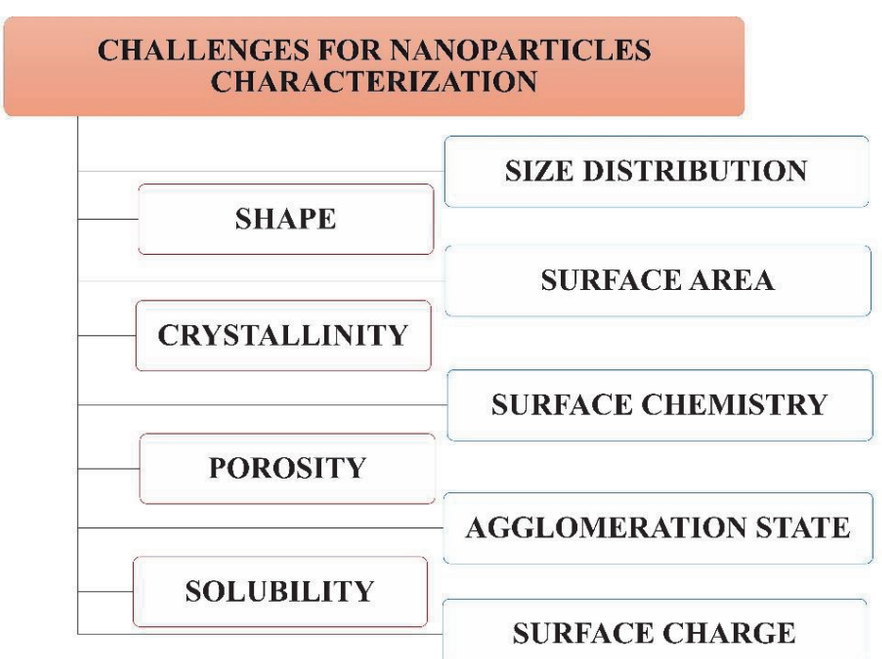

Figure 3.
Challenges of characterization of nanoparticles.

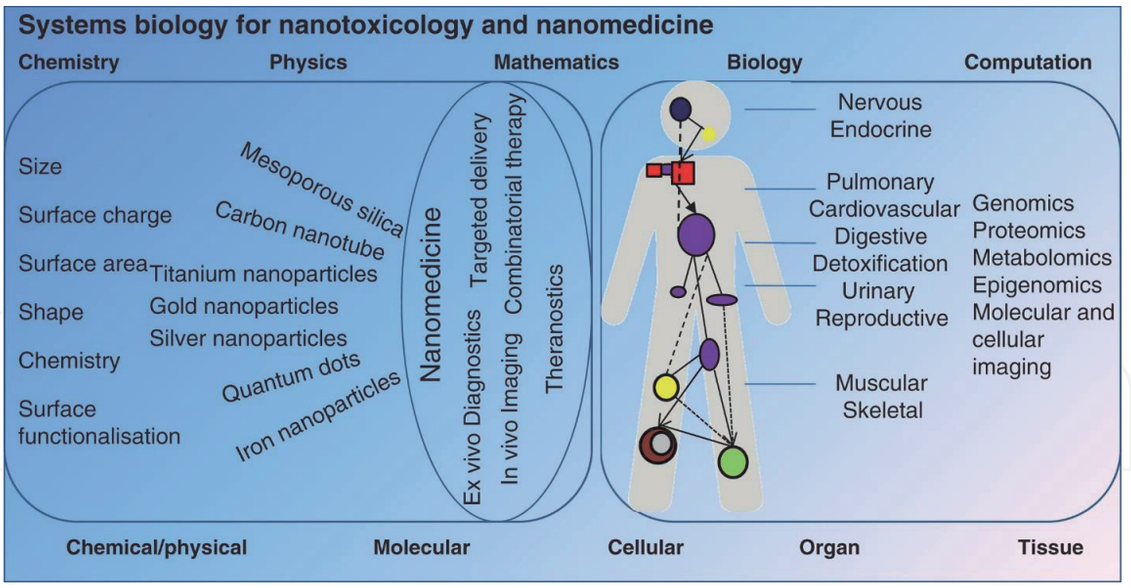

Figure 4.
Safe application of nanomaterials in therapeutics requires a deeper understanding of the material properties and behaviors at different levels of biological organization; increasing insight necessitates cross-disciplinary research collaborations (©2017 her majesty the queen in right of Canada. WIREs Nanomedicine and Nanobiotechnology published by Wiley periodicals, Inc.) [38].

nanomaterials and thus higher reliability of the results. Characterization of nanomaterials requires highly sophisticated instruments and skilled human resources to study them. The precise properties of nanoparticles and their toxicity details are poorly understood. Thus, a more wide-ranging and comprehensive characterization, including size distribution, shape, surface area, surface chemistry, crystallinity, porosity, agglomeration state, surface charge, solubility, etc., is suggested for nanomaterials in order to determine the perfect connection between their physicochemical properties and the biological effects they produce [37]. However, due to limited facilities in lab, scientists are bound to utilize the techniques available to them. Therefore, characterization techniques plays crucial role in experimental findings of nanomaterials.

On account of toxicity assessment, size is one of the crucial factors which alter the functionality of nanomaterial along with diversified interaction with living system. Size of nanoparticles can be determined by several techniques such as Brunauer–Emmett–Teller (BET), dynamic light scattering (DLS) and transmission electron microscopy (TEM). Nevertheless, further challenge is to find out accurate average sizes and size distribution which are in fact different provided by different methods. It is due to different principles involved in the several techniques. Additionally, measurement differences can also be explained based on sample preparation methods and instrument functioning procedures. However, this may generate misperception to find out the correct nanoparticle size and size distribution; therefore, one has to be well competent in the principles and technical details of the measurement methods involved. However, a deep understanding needed of NMs toxicity and their interactions with biological system **Figure 4**.

3. Assessment of nanomaterial toxicity via *in-vivo, in-vitro*, and *in-silico* approaches

The route followed by nanomaterials inside the organisms and their persistence as well as their assimilation pathways determined with a deeper understanding of the nature and interactions of NPs. There are numerous pathways to find out NPs

route as well as their affirmative parameters inside the body of organisms. Broadly, these analyses framed under *in-vivo, in-vitro*, and *in-silico* assessment (**Table 3**).

3.1 *In-vitro* methods

The *in-vitro* techniques for toxicity assessment are considered to be the most reliable, cost-effective, wider applicability, a broad range of accessibility, and more ethical due to animal fewer studies. The techniques based on the principle of

Technique	Assessment details	Instrumentation	References
In-vitro	• Selection of cell lines such as phagocytes, hepatic, hematologic, epithelial, and tumorous, etc. • Cytotoxicity assays based on ROS production, detection, and effector, etc. • Cell viability assays • Cell stress assays such as gene expression, an inflammatory marker, cell visualization, etc. • Disadvantages like lack of secondary inferences of NMs and unrevealed physiological pathways.	• Electron microscopy (SEM, TEM, etc.) • Optical spectroscopy • Dynamic light scattering	[39–43]
In-vivo	• Intracellular behavior of NMs is different and may affect various organs which mainly include: hematological toxicity, nephrotoxicity, hepatotoxicity, pulmonary toxicity, and splenic toxicity. • Studies crucially dependent upon size, surface charge, surface coating, and shape of nanoparticles. • Model living animals such as mice, zebrafish, rodents, and non-human primates • Disadvantages include non-ethical nature and a more extended assessment period	• Electron and optical microscopy • Magnetic resonance imaging • Atomic force microscopy	[41, 44–47]
In-silico	• Computational simulation and assessment of the relationship between physicochemical properties and nanotoxicity • Models illustrating nano-bio interfaces • Hazard control and risk assessment of NMs. • Development of High throughput screening (HTS) data and Quantitative structure–activity relationship (QSAR) models. • Generated data set depend upon reliable experimental toxicity results obtained through in vitro and in vivo studies.	• Theoretical calculations and computational simulations are needed to generate reliable data sets for comparative studies	[48–52]

Table 3.
Summarizes the assessment of nanomaterial toxicity via three different approaches in-vivo, in-vitro *and* in-silico.

mimicking cellular components and predicting results concerning the body of an organism [53]. The reviews are extremely helpful in regulating the dosage limits and fate of xenobiotic exposed. The different cell lines in a suitable environment exposed to nanomaterials and after incubation, the proliferation and metabolism of an exposed component are assessed with the help of different assays [54, 55]. However, physiological outcomes and prediction of results of xenobiotics are very critical. Still, primary assessment follows *in-vitro* procedures because of minor hurdles and easy availability.

Fast and comprehensive detection using in vitro technique (**Figure 5**) is proved to be the most widely accepted methodologies and various assays used in cytotoxicity investigation. The assays are different only in their mechanism of cell death and detection methodology [53, 57].

3.2 Common assays for *in-vitro* toxicity assessment

3.2.1 MTT assay

Viable and non-viable cells due to their metabolic activities releases enzymes which can further form complexes with dye molecules are the basis of colorimetric determination of cytotoxicity caused by nanoparticles [58]. The cytotoxicity assessment by analyzing the mitochondrial activity performed using MTT assay. MTT is (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium) cation (MTT+) an useful redox indicator in pharmacology [59]. The colorimetric assay based on metabolic activities of viable cells [60]. Along with mitochondrial activity, MTT assay also applicable to non-mitochondrial enzymes and endosomes, etc. The MTT tetrazolium salt crosses the membrane of active cells and reduces to formazan (1-[4,5-dimethylthiazol-2-yl]-3,5-diphenylformazan) which is a purple-colored product. The colored solution further analyzed with the help of spectrophotometry.

Figure 5.
Validated in-vitro *techniques used [56].*

The color intensity is proportional to the concentration of living cells; hence, the quantitative determination of viable cells can be completed with the help of this assay [61]. Further modification of this test leads to formation of tetrazolium derivatives such as 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT), salt (WST-1), 2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt (WST-8) which form water-soluble formazan while interacting with cells [62–64]. However, the reports show some unmatched results such as more viable cell count even at high exposure of toxic nanomaterials. Braun et al. [61] reported the overestimation of cytotoxicity at a moderate concentration of mesoporous silica nanoparticles with MTT assay when compared with ATP based assay.

3.2.2 LDH leakage assay

Lactate dehydrogenase (LDH) is a cytosolic enzyme present in all living cells. When a breakdown of the cellular membrane occurs due to nanoparticle toxicity, LDH oozes out to extracellular space where it can indicate the cytotoxicity. The free LDH in extracellular space catalyzes the interconversion of pyruvate to lactase and β-nicotinamide adenine dinucleotide (NADH) to NAD^+. Since NADH has absorbance at 340 nm, the concentration of LDH level can be determined by decreased concentration of known initial concentration of NADH and lactate [65]. Also, enzyme diaphorase utilizes NADH and H^+ for catalyzing the reductive conversion of tetrazolium salt to a highly colored formazan salt which can be measured spectrophotometrically. Wang et al. [66] reported the LDH assay for cytotoxicity determination of single-walled carbon nanotubes (SWCNTs) and oxidized SWCNTs. The formazan concentration decreases with increasing concentration of SWCNTs elucidated from spectrophotometric determination, where absorbance at 490 nm. Each cell type has specific LDH pool and passage; therefore, test measurements were first standardized with purified LDH and then LDH derived from lysed DH-82 cells were tested. Nanoparticle toxicity can affect the activity of LDH by dynamic adsorption of LDH on nanoparticle surface leading to inactivation. Also, NPs can generate free radicals or metal catalyzed oxidation processes for inactivation of LDH.

3.2.3 Trypan blue dye +assay

Trypan is an azo dye and used to stain non-viable cells and used in the cytotoxic assessment. The viable cell resists uptake of trypan and cytoplasm of these cells remain unaffected while trypan treated non-viable cells show blue cytoplasm and colorimetric determination of these cells possible.

3.2.4 Apoptosis assay

Apoptosis is programmed cell death and categorized under type-I cell death. The cell death controlled by various type of cell signals, where, a sudden stop of these signals triggers cell death. The apoptosis activation starts the initiation of extracellular proteases called caspases. These proteases further initiate activities leading to cell death. Apoptosis is characterized by condensation of chromatin and nucleus as well as DNA fragmentation. There are various assays for determining apoptosis such as TUNEL, Lamina-B, and, Apostain techniques. TUNEL is terminal deoxynucleotidyl transferase dUTP nick end labeling technique which detects the fragments of DNA which produce in the final step of apoptosis. Mechanism of the TUNEL technique involves the fluorescent dye coupling with dUTP nucleotide

present in assay, which further fastens with fragmented DNA. The quantitative analysis with the help of fluorescent microscopy or immunohistochemical staining can be done. Despite being a cost-effective and smooth operation of this technique, this technique does not distinguish necrosis and apoptosis while observing only the end stage result of the process. Apostain technique is associated with early detection of caspase-3 in the cytoplasm and does not rely on fragments of DNA. Hence, this technique is useful in early detection where activation of apoptosis and release of specific protease lead to brown coloration, and healthy cells remain blue when observed under the light microscope. This technique is particular, sensitive, and remains one of the most used methods in apoptosis analysis. Unlike apostain, Lamina-B is also an early-stage assessment technique. The nuclear lamina is the structures responsible for DNA replication, even for the reorganization of chromatin, and present in nuclear membrane. This lamina is of two type lemin-A (acidic or neutral) and lemin-B (neutral). The release of caspase −6 during apoptosis leads to lemin cleavage, which further triggers the chromatin condensation. Immunohistochemistry antigens markers are used to identify lemin-B.

3.2.5 2′, 7′-dichlorofluorescein diacetate (DCFH-DA) assay

Reactive oxidative species (ROS)induces the oxidative stress to the living cells due to internalization. The Injured cells membrane is porous for entry of non-polar dye 2,7-dichlorofluorescin diacetate (DCFH-DA) and converts into non-fluorescent DCFH due to hydrolyzation of intracellular esterase. The DCFH oxidized to fluorescent dichlorofluorescein in the presence of ROS. Thus, the quantification of ROS can be measured with fluorescence intensity measurements.

3.2.6 Comet assay

This assay named after its visual appearance, which looks like a comet, consist of single-cell gel electrophoresis technique (SCGE). This assay is widely used in vitro analysis technique, which is most reliable and inexpensive. The DNA damage during nanoparticle toxicity analyzed in this technique, whereas negatively charged DNA fragments separated using gel electrophoresis. Cells with toxicity encapsulated in agarose gel further lysed with salts and detergents which digest cytoplasm and other cell components except for nucleoids. Further electrophoresis at high pH results into a comet-like structure where the head of the comet represents intact DNA and tail comprises of the fragmented portion. Hence, the fluorescent marking and intensity of the tail show the damaged part of DNA leading to the estimation of toxicity.

3.3 *In-vivo* methods

These methods retain their most favorable and primary standards for assessment of toxicity. These studies based on the use of living animal, which is considered a little less ethical. The mode of in vivo studies involves the administration of nanomaterial into the body of testing animal and monitoring the signs of progress through different techniques. Since this procedure requires real-time analysis, and result obtained are more coherent with human body functioning, minimizing the impact of time and cost.

The *in-vivo* results for toxicity assessment are different from in vitro counterparts because of various crucial factors, which cannot involve in *in-vitro* assessment. The impact of hormonal changes, cell–cell and cell-matrix interactions add on *in-vivo* assessment. The long-term chronic effects are not possible *in-vitro* studies; hence, some impacts are missing during *in-vitro* analysis. The in vivo studies,

however, carried out with more considerable precautions because they are interlaced with many challenges. *In-vivo* dose is determined based on actual exposure of nanomaterial to the body, which is a technical challenge because of minimal size and peculiar properties in the biological system. During *in-vivo* experiments, the vehicle to carry out nanoparticle dose must be non-reactive, and NPs must disperse appropriately in it. Since NPs are very susceptible to agglomeration due to their larger surface area. Agglomeration and poor dispersion lead to improper biological distribution and unwanted results. Once the nanomaterial inside the body, they can interact with protein counterpart leading to the formation of the protein corona. These lead to alteration in the properties of NPs, their interaction, and biodistribution. Protein structure further undergoes conformational changes and leads to modified biological functions as well as altered signaling pathways. Hence before assessing the toxicity of NPs in a biological system, one must also consider the various interferences of NPs with another substrate [67].

Chen et al. [68] investigated gold nanoparticles (AuNPs) of size 21 nm on male C57BL/6 mice by collecting the tissues after 1, 24, and 72 h post injecting the 7.85 μg AuNPs/g solution of AuNPs. Further analysis was done using Scanning Electron Microscopy (SEM) and proinflammatory cytokine expression, as well as macrophage counting, was done with real-time PCR. The results show the compatible nature of AuNPs with living tissues and not observed a significant change in the number of macrophages. However, the reported results show an accumulation of AuNPs in abdominal fat, and some quantity also found in the liver, leading to a reduction of fat in AuNPs treated mice. Rizzo et al. [69] used zebrafish embryo for correlating the results obtained from in vitro analysis with in vivo studies. Authors used different NMs for toxicity assessment both in vitro assays. The coating on nanomaterials with biocompatible polymers shows a significant decrease in the toxicity. The results for pristine ultra-small superparamagnetic iron oxide (USPIO) and flavin mononucleotide coated USPIO (FLUSPIO) and sineram tested in vitro on HeLa (human cervical carcinoma), HUVEC (human umbilical vein endothelial) and SMC (ovine smooth muscle) and in vivo studies carried out on zebrafish embryo assay. The in vitro studies do not show any cytotoxic effect on different cell lines up to concentration 10 mg/mL, on the other hand in vivo studies for toxicity analysis on zebrafish embryo assay show different results as compare with in vivo. The similar dose of NP causes lethal effect on embryo. The toxicity of pristine USPIO greater than coated counterparts, FLUSPIO and sineram. Even the lethal effect not observed for coated nanoparticles at high exposure time up to 72 and 168 h. The probable reason for cytotoxic effects given by authors was aggregation of uncoated nanoparticles and further due to larger hydrodynamic size lead to blockage of egg chorion pores [70]. In another study based on zebrafish embryo shows stage-dependent toxicity and specific phenotype with AgNPs (97 ± 13 nm). Different developmental stages of embryos have different critical concentration of nanoparticles such as Cleavage stage (3.5pM), Gastrula stage (4pM), Segmentation stage (6pM), Hatching stage (8pM) [71]. The maximum number of abnormalities found in deformed zebrafish developed from cleavage and gastrula stage of embryos. However, the later stages do not show significant deformities. The early-stage embryos show head and eyes deformities which are not present in later-stage embryos. The cleavage stage and gastrula stage abnormalities are more prominent and also increases with increase in concentration of AgNPs owing to their impact on early determinative events like cell signaling and gene transcription. The AgNPs stays inside embryos throughout their development. The longitudinal thin layer sections with all deformities shown in **Figure 6**. The observed NPs found embedded in eye, pericardial space, and in tail which are characterized by LSPR spectra of individual AgNPs.

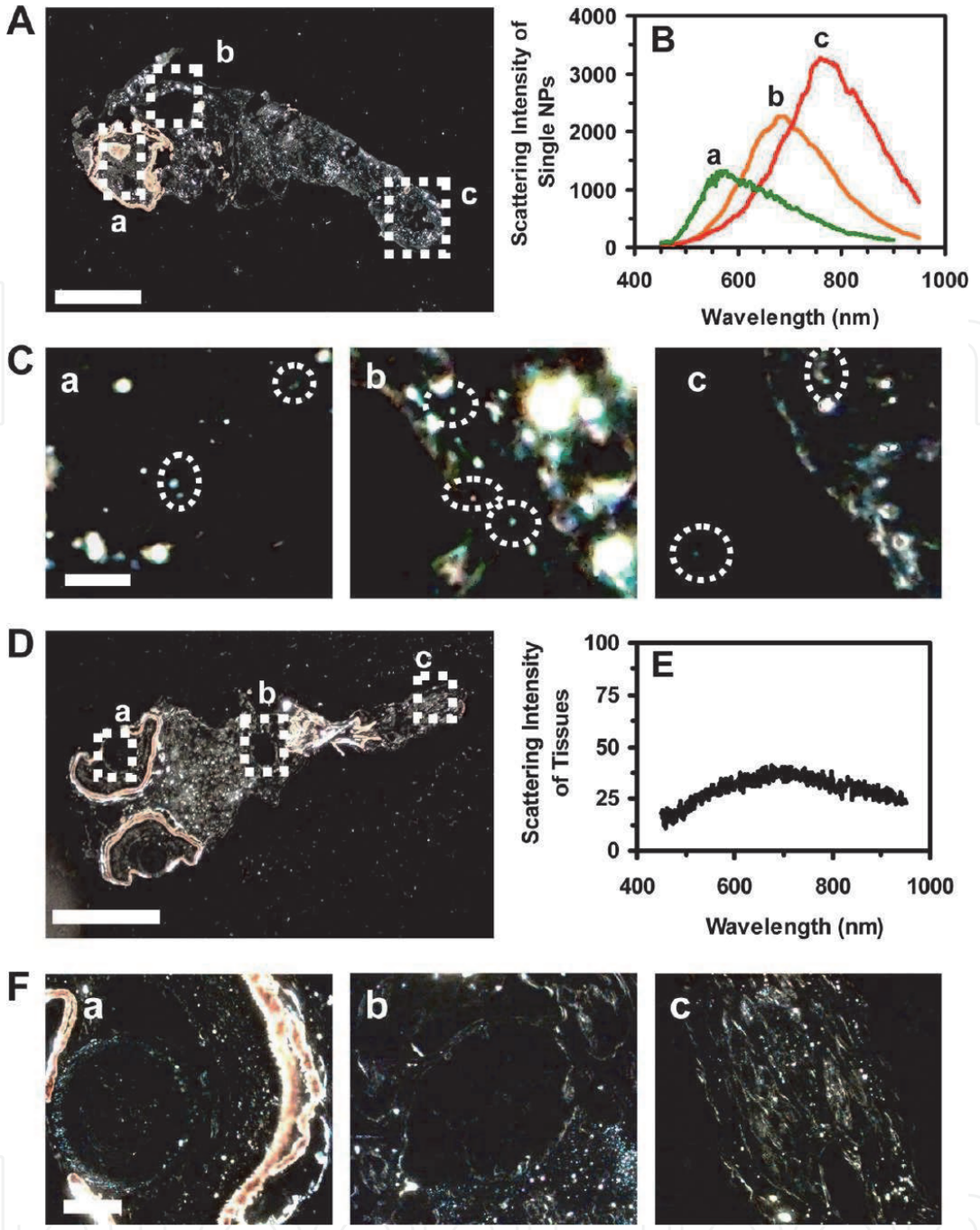

Figure 6.
Imaging and characterization of individual Ag NPs embedded in the tissues of (a – C) deformed zebrafish and (D – F) normal zebrafish (control) using DFOMS-MSIS. Optical image of thin-layer longitudinal section of fixed (a) deformed zebrafish with five types of deformities and (D) normal zebrafish. (C) and (F) zoom-in optical images of the tissue sections of (a – c) as highlighted in (a) and (D), respectively: (a) eye (retina), (b) pericardial space, and (c) tail. (B) LSPR spectra of individual Ag NPs as circled in (C) show distinctive λ_{max} (fwhm): (a) 567 (176), (b) 688 (185), and (c) 759 (179) nm. (E) Scattering intensity of the tissues of normal zebrafish in (F) shows the background (nondistinctive plasmonic colors). Scale bars in (a) and (D) are 250 μm and in (C) and (F) are 5 and 30 μm, respectively. "Reprinted with permission from [71] (2013) American Chemical Society.

3.4 *In-silico* assay

Considering the time requirement, ethical standards, and reliable results, scientist prompted to use alternative ways for analyzing the toxicity of materials. The *in-silico* analysis one of the novel approaches as compared with general studies. The procedure is based on the principle of theoretical modeling and simulation of results for various physicochemical properties of molecules (**Figure 7**). The available data

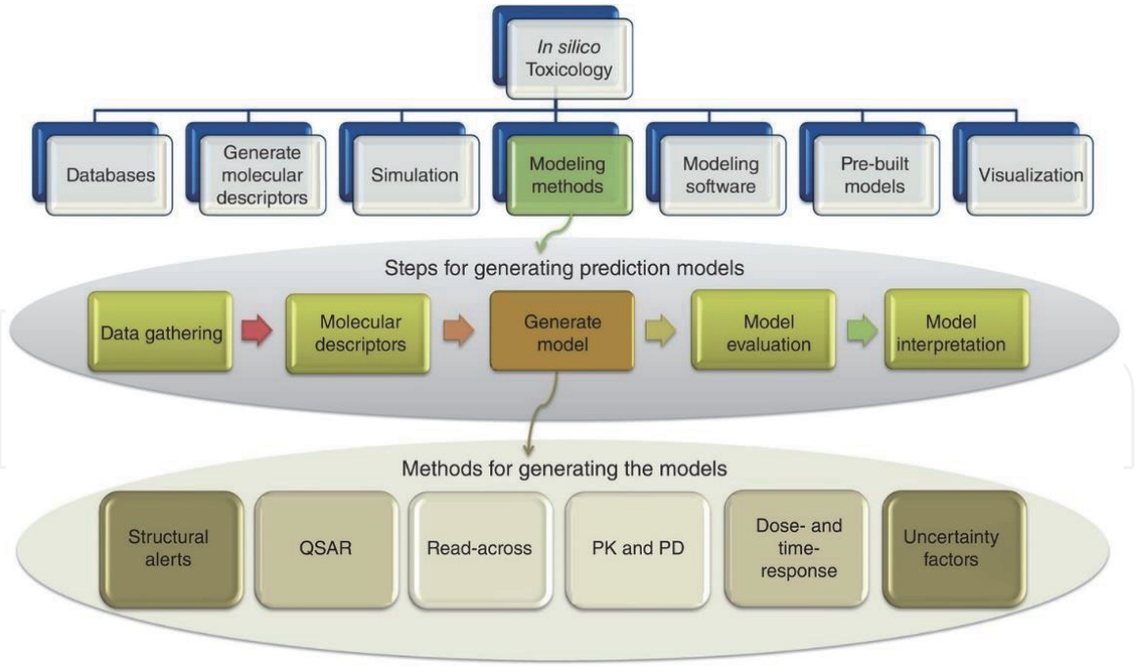

Figure 7.
In-silico *toxicology tools, steps to generate prediction models, and categories of prediction models (copyright © 2016 the authors. WIREs computational molecular science published by John Wiley & Sons, Ltd. [73]).*

of toxicity of material and their interpolation using multiple mathematical models, in silico studies owes many advantages still there are limitations because experimental verification needed additionally to prove the toxicological effects. Also, due to the data gap, the quantitative risk assessment of nanomaterials on web-based tools has not much explored. Current methodologies based on exposure assessment in production and manufacturing life stages while ignoring the exposure during use and end stages of the life cycle of nanomaterials. Based on physicochemical properties and their descriptions, computational chemistry methods has been modified to nano-based models such as nano quantitative structure–activity relationship (nano-QSAR) or quantitative nanostructure activity relationship (QNAR) [73].

In-silico methodology selects the models that have historical development or represent state-of-the-art methods for assessment of toxicity. Structural alerts and rule-based models are used for evaluation of toxicity. The structural alerts are chemical structures representing the toxicity while rule-based models are derived either from human knowledge and literature (Human-based Rules) or from computational simulations of data (Induction-based Rules), which rely on probabilities [72, 74].

Two European projects named GUIDEnano tools and SUN Decision Support System (SUNDS) provides valuable information about the implementation of tools for assessment of nano-enabled products in their whole life cycle [75, 76]. These web-based tools create a sustainable portfolio for production, handling, and end cycles of engineered nanomaterials. It also needs the exploited data about physicochemical, toxicological, and exposure of nanomaterials. Life cycle analysis approach critically required for assessing the impact of nanomaterials on the environment. The collection of data, transfer, and transformations of nanomaterials, Leading to toxicological effects to humans and environment can be predicted through risk assessment tools.

The above-discussed assays, however, experience production of erroneous results due to interference arising due to NPs solubility, agglomeration, particle sedimentation, and, the formation of the protein corona. The problem can be

appropriately acknowledged by designing and establishing a standard set of essays which need to be following particular nanotoxicological standards and uniform applicability. The generation of standardized protocol further faces the challenge of different nature of nanomaterials to be assessed since metallic NPs, and carbon-based nanotubes have different physicochemical characteristics in physiological conditions. The metallic NPs and CNTs show different physical traits at nano-cellular interface leading to altering the biological response. CNTs and metallic NPs both produce ROS species but follows different pathways where metal NMs causes apoptosis, while CNT leads to fibrosis and inflammation [77]. CNTs and metallic NPs are also different in their assimilation pathways in the biological system. CNTs found to be less biodegradable and persisting in system for a longer duration while, metallic NPs undergo dissolution into ions further disrupting the biological pathways [78, 79].

3.5 Physicochemical parameters for toxicity assessment

Cellular responses are directly linked to physicochemical parameters of nanomaterials. The advancement in material science has achieved a precise synthesis of nanomaterials with adjustable target and specific action. There are various morphological factors on which nanotoxicological response depend such as size, surface morphology, charge and, composition, etc.

3.5.1 Size of NMs

Size of the NMs are a primary factor for determination of cytotoxic response. It affects the internalization process into cells and endocytosis process, which ultimately altering the intracellular fate of nanomaterials. Most of the studies conclude that smaller the size of NPs, higher the degree of cytotoxicity [80, 81]. Bharadwaj et al. [82] reported the assessment of variable sized nanoparticles ranging from 20 to 500 nm in processed brain tissue sections with the help of confocal microscopy. Maximum accumulation was observed for 1 hour and it was found that 500 nm particles accumulated the most. However, NPs shows selective accumulation behavior. In the case of quantum dots (QDs), the cytotoxicity and size depend upon the method of preparation. QDs produced by using ligands like trioctylphosphine lead to hydrophobic nature and further converted to hydrophilic, leading to an increase in hydrodynamic radius of NMs [83].

3.5.2 Surface

Cellular uptake mechanism and cytotoxicity relies on the morphology of NPs. NMs have different kind of shapes, which includes spheres, needles, cubes, tubes, rods, etc. Membrane interactions during internalization of NPs affect the nature of barriers. Researchers reported the formation of pores in cell membranes due to interactions of NPs, leading to an imbalance in an ionic concentration outside and inside of the cell [84]. Chithrani et al. [85] reported the effect of size and shape of AuNPs in internalization and concluded that when morphology changes from rod shape to spherical, there is an increase in uptake up to 500%. Recently, Maysinger et al. [86] also studied the gold nanourchins whose surface morphology has an irregular shape. The functionalization with polyethylene glycol (PEG) on AuNPs did not show any significant alteration in viability and morphology while cetyltrimethylammonium bromide (CTAB) modification showed adverse effects on filamentous actin and, nuclear lamina.

3.5.3 Surface coating and charge on NMs

Surface coating on the nanoparticles surface act as a connecting link between nano-cellular interfaces. Coating affects the interparticle interactions, cellular contacts, internalization, and cytotoxicity of material [87]. Surface coating possesses distinct charges which can alter the cytotoxicity of materials. Various studies show that positively charged NMs internalized more effectively and also result in more toxic effects than neutral or with negatively charged particles [88, 89]. Coatings broadly divided based on interactions into three types by Richards et al. [90]. These are covalent coatings having covalent bonding, the electrostatic surface coating having electrostatic interactions, and atomic layer deposition where chemical bond formed between molecule and coating material. Coating plays a crucial role in various nanomaterial application in drug delivery, imaging, and cancer treatment [91, 92]. Coating of chitosan reduces the production of ROS species in different CuNPs and Fe_2O_3 NPs and reduces the inflammatory response and overall toxicity of nanomaterials [93, 94]. Yin et al. [95] studied nickel ferrite NPs and explained their toxicity through their surface coating with oleic acid. The toxicity with coated nickel ferrite NPs depends upon the dose of material. The coating also shows significant effects when changed from single layer to double layer by changing hydrophobic and hydrophilic, respectively. It was observed that hydrophobic coating impart a high level of toxicity than the hydrophilic counterpart. Nanomaterials are internalized through lipid bilayer membrane structure where the charge on the membrane is negative. Hence, opposite charged NPs pass through effectively due to electrostatic interactions while negatively charged bound less efficiently. The acid treatment of carbon nanotubes (CNTs) leads to high toxicity due to surface functionalization. The negative charge introduced due to hydroxyl (—OH) and carboxylic acid (—COOH) contribute to more toxicity [96].

4. Future prospective and conclusion remarks

Nanotechnology exhibit excellent potential in developing new materials every day; hence, nanomaterial safety also deserves much attention for their safer use. Therefore, understanding the environmental fate and biological impact is highly desired for designing biocompatible materials in place of abandoning nanomaterials. There are numerous techniques to date for analyzing the toxicity caused by nanomaterials. Still, diversified nanoparticles, different behavioral impacts and variegate incubation protocols have rendered it and impossible to draw the conclusion regarding toxicity. Nanotoxicity assessment broadly carried out in two concerning fields; biological and environmental. The environmental factors such as temperature, ionic strength and transformation of NMs inside biological system regulate the toxicity. The properties of agglomeration, physicochemical changes, and nano-bio interface interactions needs pharmacokinetic studies of NMs. Most of the studies carried out with pristine NPs for toxicity assessment; however, product or degradation product of NMs enter into environment and should be encountered instead of pristine NMs. The transformed or degraded materials in environment remain major challenge toward assessing toxicity specially in case of carbon-based nanomaterials on biological substrates.

Biological fate of nanoparticle toxicity is the second major field where high-quality instrumentation, sophisticated culture medium, and reliable in vitro and in vivo assays are needed. Many recent studies accentuate in the present perspective and put forward some model system to address the critical issues and dealing with the impediment of assessment of nanotoxicity. Apart from standard viability tests,

other parameters need to be considered such as intracellular stability, degradation potential, and excretion pathways. There is a dire need for collaborative approach and multidisciplinary aspect to analyze the broad field of NPs and cell interactions. The major challenge in intracellular molecular events where relationship among biological functions need to be addressed. The recent development in assessment of toxicity include the adverse outcome pathways that include more proficient, predictive mechanistically approaches. This conceptual framework links the biological event with molecular initiating event at earlier stages [97]. Advanced electroanalytical methods can be used to monitor these events and play determinant role in assessment. In silico approach for nanotoxicity determination has also emerged out as an alternative to in vivo and in vitro analysis. The computational simulation of data, however, relies on experimental findings but more ethical mean for toxicity assessment. The characterization of nanomaterials plays substantial role in computation of engineered NMs along with establishing the relationship between biological activity and nanostructure [98]. Hence, programmatically executed reliable experimental data can be utilized to predict the nanotoxicity before their manufacture and use. In upcoming decade, Qiu et al. [99] presumed four basic predictive models for nanomaterial properties and biological effects. These analytical challenges include nanotoxicological mechanism changed from correlative to causative aspect, to conquer nanoparticle interferences for explicit in vitro analysis, establishing single-cell level cellular response for nanoparticle interaction, and understanding kinetic parameters of nano-bio interfaces.

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