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Review
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Toxicity of Nanomaterials: Exposure, Pathways, Assessment, and Recent Advances

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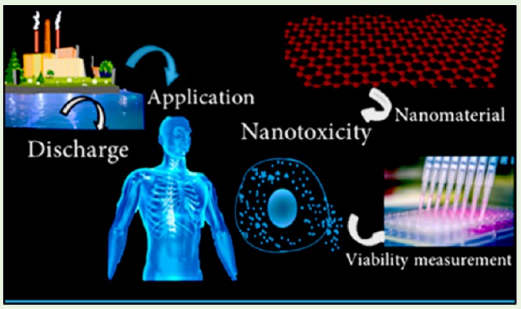
ABSTRACT: Growth in production of manufactured goods and the use of nanomaterials in consumer products has mounted in the past few decades. Nanotoxicology or toxicity assessment of these engineered products is required to understand possible adverse effects and their fate inside the human body. The present review is a one stop assessment intended to be a state of the art understanding on nanotoxicity. It provides a summation of the various kinds of cell death and also discusses the different types of toxicities along with their studies. The review discusses the physiological impact imparted on cells (reactive oxygen species generation and the resultant oxidative stress, inflammation, and other nonoxidant pathways). Moreover, it discusses the different physicochemical properties of nanomaterials (size, morphology, surface charge, and coating) governing the cytotoxicity properties. It also details the major pathways of nanomaterial uptake in cells and their outcome. Additionally, it also discusses the possible methods for human exposure to nanomaterials (skin, respiratory tract, gastrointestinal tract, blood brain barrier, liver, and spleen). Furthermore, an entire new section is contributed in discussion of all possible types of assays (cytotoxicity, cell proliferation, and genotoxicity assays). A summarized discussion of the recent advances on in vitro, in silico, and in vivo studies of nanomaterials (metal, metal oxides, carbon nanotubes, graphene, and other novel materials) is made. The review also provides a brief account of the safety guidelines for handling nanomaterials. Finally, the uses of engineered nanomaterials in commercial products are discussed in detail.



KEYWORDS: *cellular uptake mechanism, physiological end point, toxicology, nanotoxicology, human exposure, 2D layered materials*

INTRODUCTION

In the current landscape, manufactured goods without some component of nanomaterials (NMs) are very rare.[1,2] NMs are materials with dimensions typically less than 100 nm; Figure 1 gives an overview of the size profiles of different materials.[3,4] Famous physicist Richard Feynman envisioned a quantum world long before the discovery of a diode, where he imagined writing an entire volume of Encyclopaedia Britannica on the head of a pin.[5] Since then, development of analytical tools and experimental processes have provided us with the armor to build such a quantum world.[6]

All living bodies are constantly exposed to foreign materials (xenobiotics) such as nanomaterials in one way or another.[8] The use of manufactured products incorporating nanomaterials has risen exponentially in the previous decade and is believed to increase up to 58 000 tons by 2020.[9,10] The boom in these nanomaterial industries can be imagined by the amount of money invested by United States government for the financial year 2016−2017.[11,12] The National Nanotechnology Initiative (NNI) is planning to spend $1.4 billion on research projects to comprehend the transport and life cycle of nanoparticles (NPs) in the earth and in this way evaluate its impact on people and the environment.[13,14]

Nanomaterials of all dimensions (0D, 1D, 2D, and 3D) are used extensively in various applications such as the use of metal nanoparticles for groundwater remediation; arsenic removal and groundwater treatment using iron oxide nanoparticles; titania nanoparticles for sunscreens and paints; fullerene nanotubes for tennis rackets; video screens; silica nanoparticles in electronic industries; zinc oxide nanoparticles used as potential industrial coatings to screen from UV rays on wood, plastics, and textiles; silver nanoparticles as a potential antimicrobial agents; use of graphene and carbon nanoparticles as electrodes in fuel cells; etc (Figure 2).[10,15−29]

Apart from all the hype associated with nanomaterials, there remains a definite ambiguity over their physicochemical effect on living beings.[30,31] Analyzing the effect of nanomaterials on the environment is very challenging as it depends on an intricate set of factors such as size, shape, surface properties, charge, etc. of the nanomaterial.[32−36] However, as with any other contaminant, the impact on the environment depends on its physicochemical properties which influence its surroundings as it

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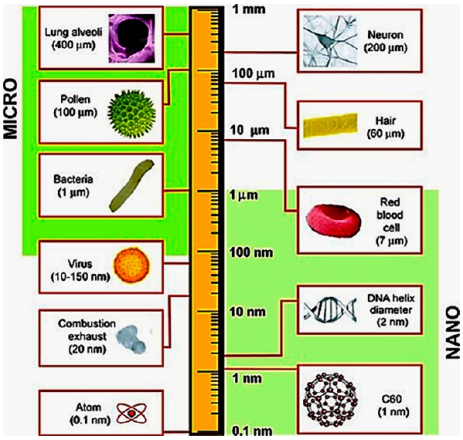

Figure 1. Schematic illustrations of size profiles of different materials. Reproduced with permission from ref 7. Copyright 2007 American Vacuum Society.[7]

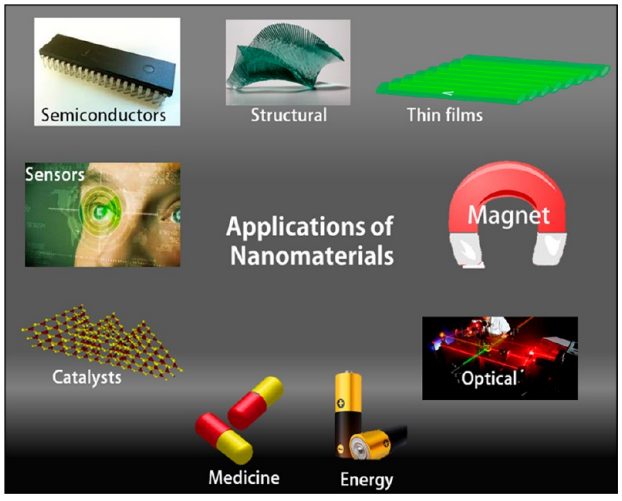

Figure 2. Applications of nanomaterials.

migrates and transduces.[37] The fate of these nanomaterials inside living cells has been evaluated for a long time, but there exists a definite paucity of the basic understanding of toxicity and establishing a general principle to evaluate all the materials under a common domain.[38,39] Extensive research is being conducted around the globe on different nanomaterials to alter their behavior in any form (morphologically, optically, or chemically). However, the scientific understanding of their impact on living bodies is left out. A single variation in the nanomaterial can lead to a complete change in its behavior and so does its mode to impact living cells. Even in the presence of state of the art toxicity evaluation methods, the constantly evolving material synthesis process and complex material characterization poses a great task. There exists some research demonstrating the impact of nanomaterials in creating diseases such as asthma, dermatitis, rhinitis, pleural, interstitial lung disease, lung contaminations, tuberculosis, respiratory embolism, bosom malignancy, lung growth and immune system illnesses, and so forth. Therefore, the ultimate understanding of nanomaterial toxicology (nanotoxicology) is imperative in this current scenario. A systematic understanding of nanotoxicity can help researchers to pick materials which are environmentally benign and prioritize research to mitigate credible risks associated with the environment and human health.[37,40,41]

Reviews detailing only the diverse types of assays for toxicity estimation and articles solely dedicated on discussing only the toxicity studies of different nanomaterials have been widely published in the last few decades.[42−48] However, the present review aims to provide an overview of all the possible dimensions of nanotoxicology. It is aimed to deliver an overall insight to a new researcher about the past, present, and outlook of nanotoxicological research. It discusses the various kinds of cell death and toxicities. A brief discussion of the factors affecting the physicochemical properties of nanomaterials and their physiological impact on the cell is included. The review also summarizes the possible pathways of nanomaterial exposure to the human body and the potential routes for uptake inside the cell. A major section has been assigned to discuss the diverse types of toxicity assays. An account of the recent advances of in vitro and in vivo toxicity studies of nanomaterials is provided. Finally, the review provides a brief explanation of the safety guidelines for handling nanomaterials and the commercial products utilizing engineered nanomaterials.

WHAT IS NANOTOXICOLOGY?

The term nanotoxicology may raise an alarm among the general population; however, it is a moderately new branch of toxicology that addresses the gap in knowledge of toxicity induced by nanomaterials. According to Donaldson et al., this new realm of toxicology introduces protocols to access the toxicity induced by nanomaterials. This branch includes the basic understanding of the physicochemical effects of nanomaterials and their routes of exposure/uptake mechanisms for toxicity assessment in humans and the environment.[49] Toxicity assaying might not be a new topic of concern, but the use of nanomaterials in a rapid score has shifted the paradigm toward nanotoxicity assessment. He et al. in a more recent review discusses the importance of the nanobioeco interface. The nanomaterials when discharged in the environment might undergo modification which could eventually increase or decrease the toxicological profile of these materials. Hence, the dynamics of the environment introduces an uncertainty toward the fate of the nanomaterials.[14] Figure 3 exhibits a schematic of different pathways leading to the nanobioeco interface.

Figure 3. Schematic of different pathways leading to the nanobioeco interface. Adapted with permission from ref 14. Copyright 2015 The Royal Society of Chemistry.

Growth Phase of Cells. To understand the cytotoxicity effect on cells, it is essential to understand the growth phases of them. The standard growth rate curve is very important to understand the growth mechanism of different cell lines. These growth rate curves help to estimate the difference in the cytotoxicity effect and evaluate the cell growth with time or mass. A growth rate curve is defined as the logarithmic of the number of cells

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with time. There exist four basic growth phases such as lag, log, stationary, and death phase (Figure 4).[50−52]

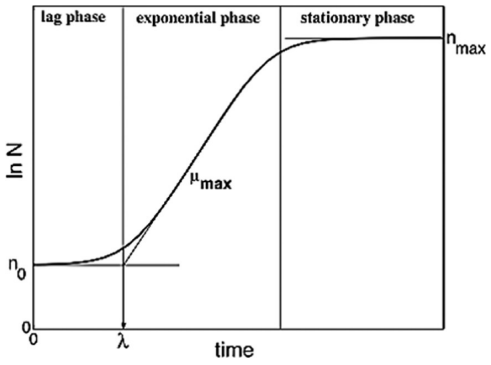

Figure 4. Microbial growth rate curve where n_0 is the initial population density and n_{max} the final maximum population density. Reproduced with permission from ref 53. Copyright 2004 Elsevier.[53]

Lag Phase. The stage in which the multiplication of new cells does not happen. In this stage, the cell experiences high metabolic action to synthesize enzymes for reproduction activity. The cells undergo necessary adaptation required to exploit the fresh environmental surroundings. This procedure could comprise the repair of macromolecular impairment added during the stationary phase.[52−54]

Log Phase. This stage is otherwise called the exponential development stage. In this stage, the metabolic action stays steady, yet the multiplying or the propagation of new cells increases in an exponential way and reaches a maximum growth rate (μ_m) in an unequivocal period (λ). The cells in this phase are very sensitive, so any sort of interference such as radiation, drugs, nanomaterials, and so forth can harm cell growth.[52−54]

Stationary Phase. In this phase, the growth rate decelerates, and concurrently, the metabolic activities of the cells reach an equilibrium level. The growth rates are controlled by the increase in the death of cells. Therefore, in this phase, the total cell growth achieves a stationary point, and the curve attains an asymptote (A).[52−54]

Death Phase. The development of new cells is surpassed by the decease of the cells in this stage. The death of the cells continues until all the cells are diminished completely.

Various Kinds of Cell Death. To put nanotoxicity studies in context, the present section aims to provide a brief discussion on the several types of mammalian cell death. Cell death is a normal cellular occurrence, operational in the body to attain regular metabolic activities. However, diseases are triggered due to the death of either modest number of cells or their death in abundance. Considering the morphologies of mammalian cells, the cell death mechanisms are divided into three types: apoptosis, autophagy, and necrosis.[55]

Apoptosis. Apoptosis is otherwise called the type-1 cell death, which is controlled by various sorts of cell survival signals. Missing signals or the sudden stop of these cell survival signals often triggers apoptosis. Activation of apoptosis results in initiation of a few intracellular proteases called caspases. There are 14 distinct sorts of caspases known in a mammalian body. These proteases initiate a series of activities which further stimulate the cellular death mechanism. For example, caspase-3 initiates CAD/DFF40, a DNase that destroys DNA and nuclear material. The distinctive property of this type of cell death is the obliteration of nuclear morphology, chromatin condensation, disintegration, and development of apoptotic bodies or fragments (Figure 5).[56−60]

Autophagy. Autophagy, an intracellular degradation system, is otherwise called type-2 cell death. It is described by double membrane formation inside the cell (Figure 6). It is a nonparticular degradation mechanism yet has a few pathophysiological significances; for example, starvation adaptation, intracellular protein and organelle clearance, elimination of microorganisms, cell death, tumor suppression, and antigen presentation.[62,63] Nutrient starvation inside the cell, caused during cell division, differentiation or various forms of external

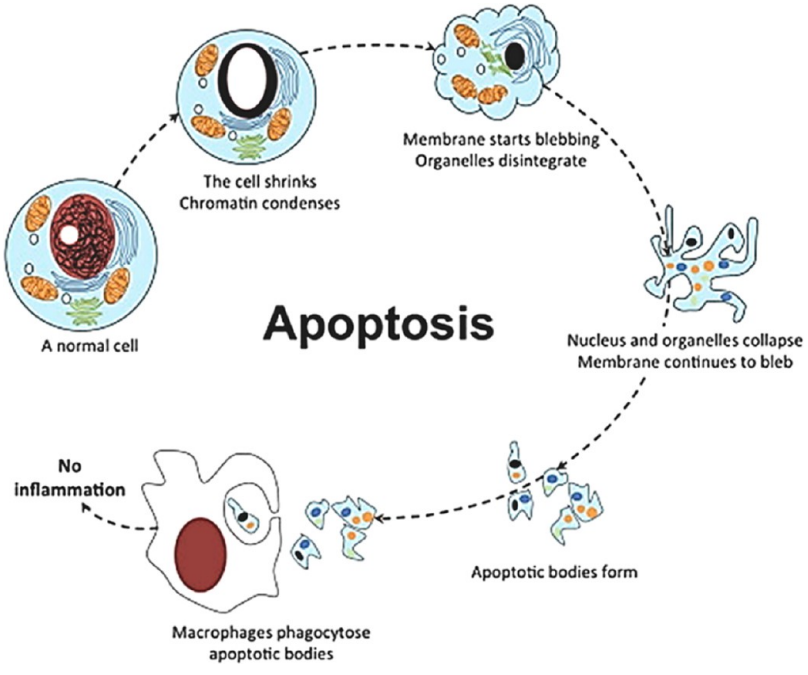

Figure 5. Apoptosis cell death mechanism. Reproduced with permission from ref 61. Copyright 2015 Elsevier.[61]

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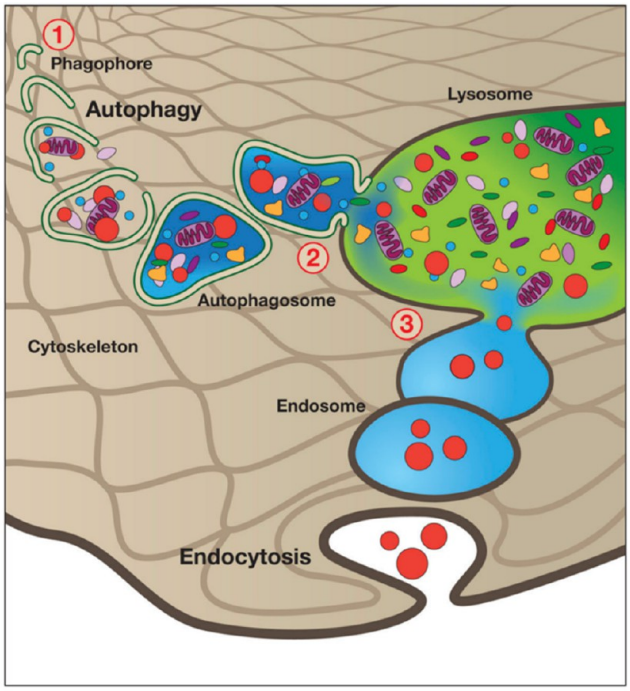

Figure 6. Autophagy cell death mechanism. Reproduced with permission from ref 68. Copyright 2012 Stephan T. Stern, Pavan P. Adiseshaiah, and Rachael M. Crist.[68]

stress, is one of the prime reasons of triggering autophagy. The process initiates by the formation of double layered membrane containing vacuoles known as the autophagosomes. It engulfs the cytosolic materials and further fuses with lysosomes and forms autophagolysosomes to cause degradation. After the degradation of the macromolecules, the monomeric units are further reused in the cytosol.[64−67]

Necrosis. The type-3 cell death phenomenon, a result of physicochemical anxiety and so frequently referred to an inadvertent and uncontrolled cell death (Figure 7). It is initiated by

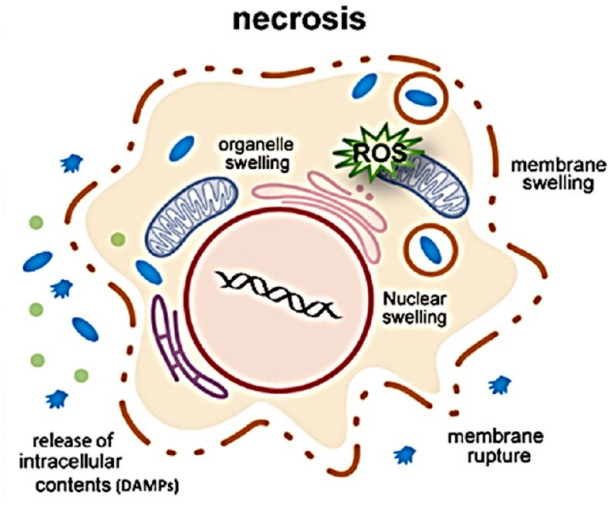

Figure 7. Necrosis cell death mechanism. Reproduced with permission from ref 72. Copyright 2014 Zong Sheng Guo, Zuqiang Liu, and David L. Bartlett.[72]

irreversible alterations in the nucleus (karyolysis, pyknosis, and karyorhexis) and in the cytoplasm (condensation and intense eosinophilia, rupture of structure, and disintegration). Necrosis and cell death are not the same singularities; the necrotic changes in the cell structure cannot be detected until 12−20 h after the cell death, and hence, necrosis cannot be termed as a proper cell death system.[69] However, the other two kinds of cell death initiate the necrosis process. Necrosis is characterized by the cytoplasmic swelling, dilation of organelles, which results in cellular vacuolation, and rupture of the plasma membrane, which leads in the proinflammatory outflow of the intracellular content. The spilling of the cytosolic content also causes damage to the cells in the surrounding.[70,71]

Distinct Types of Toxicity. *Biodegradation.* Biodegradation is a biologically catalyzed procedure of breaking natural complex particles into simpler products (Figure 8). The microorganisms participate in the disintegration process of the complex organic compounds into simpler products. Biodegradation is divided into two categories: biomineralization and biotransformation. In biomineralization process, the complex organic compounds are divided into simpler inorganic molecules such as carbon dioxide, water, etc., whereas in the biotransformation process, the compounds undergo incomplete breakdown and transform into a simpler compound, which may or may not be toxic in nature, and an inorganic molecule. The biotransformation of a few nonlethal mixes regularly winds up into more intense poisonous products. Hence, biodegradation is one of the credible routes to induce toxicity in the environment. The biodegraded compounds often wind up aggregated in the earth and ruin the normal enzymatic capacity of several organisms.[73,74]

Bioaccumulation. Uptake of xenobiotic materials inside living bodies via food or body surface is known as bioconcentration, and the resulting exchange of the foreign substance on the organisms present in the higher levels of the food chain is known as biomagnification (Figure 8).[75] The uptake of these xenobiotic materials frequently instigates toxicity in the living bodies. The idea of bioaccumulation can be best portrayed utilizing the ideal case of DDT (dichlorodiphenyltrichloroethane). DDT is widely known pesticide which has a half-life of 15 years, and therefore even after 100 years, the compound does not become completely degraded. Biotransformation of DDT frequently winds up in more poisonous exacerbates; the breakdown of these by-items is considerably even more acute. Consequently, they get amassed in the soil and water sources.[76] Microorganisms, fish, and several other living beings absorb or uptake these chemicals and subsequently get transferred into the living beings higher in the food chain. Therefore, bioaccumulation is another mode of inducing toxicity in the environment.[77,78]

Genotoxicity. Toxicity causing destruction to the genetic material of the cell is known as genotoxicity (Figure 8).[79] Materials which cause damage to the integrity of the genetic material inside the cell are known as genotoxins. Genotoxicity induces a distinct number of problems inside the nucleus of the cell. It can lead to mutation of the DNA strands such as duplication, deletion, chromatic aberrations, etc. The DNA damage may lead to malignant transformation of the cells, and in a few instances, they might cause aberration inside the germ cells, which might lead to hereditary diseases such as diabetes, cystic fibrosis, sickle cell anemia, hemophilia, etc. Various in vivo and in vitro genotoxicity assays are developed to estimate and access the risk of various materials. Therefore, genotoxicity is one of the prime routes for toxicity induction in living organisms.[80−82]

Cytotoxicity. Toxicity causing the destruction of the cell is known as cytotoxicity (Figure 8). It is one of the most

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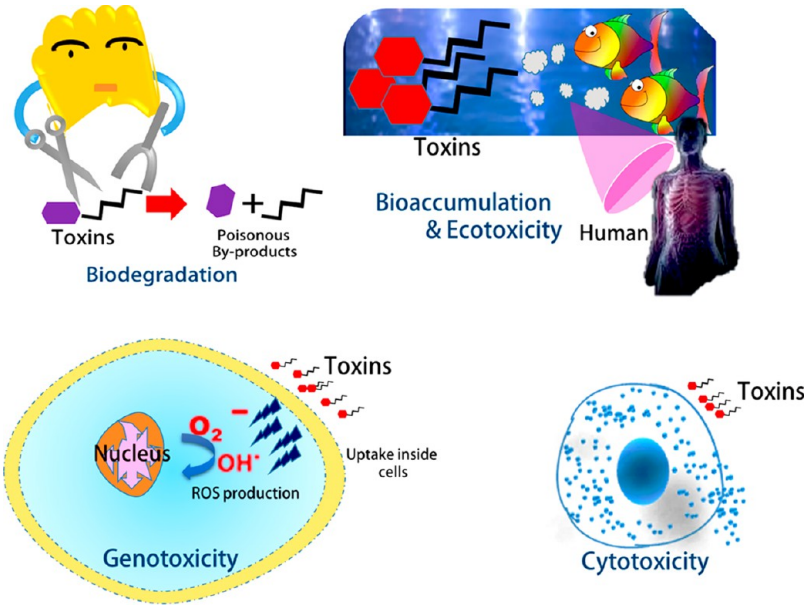

Figure 8. Schematic illustration of different types of toxicity.

predominant toxicity effects observed in living organisms. Exposing the cells with toxic compounds often leads to various fatal results such as complete breakdown, rupture of the cell membrane, and destruction of cytosolic components. They might also initiate programmed cell death phenomenon (apoptosis) which might lower the growth rate, i.e. decrease the number of cells or reduce the chances of viability or proliferation.[83]

Ecotoxicity. Toxicity is induced in the environment due to multiple reasons, but the effect of the same can be observed in the ecosystem in general (including humans). Ecotoxicity is a broader term to introduce the concept of toxicity and its effect on the entire ecosystem (Figure 8). Potential ecotoxins in the environment are man-made engineered products such as nanomaterials, hydrocarbon, synthetic inorganic molecules such as pesticides, etc. These potential toxic compounds further enter the food chain system through bioconcentration and proceed upward in the higher order animals through biomagnification (as discussed earlier above). These toxic effluents affect the cell and genetic materials of the living organisms to impose cytotoxicity and genotoxicity in them.[32,84,85]

Type of Toxicity Studies. *Acute Toxicity.* Acute toxicity is defined as the hostile changes observed after a short time of administration (within 24 h) of single (or multiple) doses of substance. An adverse effect is defined as "some effect that leads to functional damage and/or biochemical abrasions that may affect the functioning of the entire organism or that decrease the organ's capacity to react to an added encounter". The acute toxicity assessment is used to evaluate the dose-dependent effect of the substance on the organism at the different time of exposure. This toxicity study helps in median lethal dose (LD_{50}) and effective dose (ED_{50}) tests. LD_{50} is the effective dose which kills 50% of the population exposed (Figure 9). In contrast, ED_{50} is the effective dose which causes any specific effect other than lethality. It is used to determine the therapeutic index and it is the ratio between the lethal dose and the pharmacologically effective dose (LD_{50}/ED_{50}). The higher the index, the lower the toxicity of the substance and vice versa.[86,87]

Subchronic and Chronic Toxicity. The chronic tests study the adverse effect on two species (one rodent and one nonrodent) and dosed daily for six months. In the case of subchronic tests,

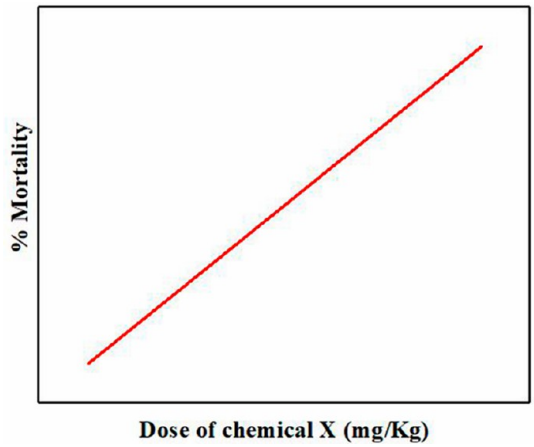

Figure 9. Percentage mortality plotted against the increasing dose of the chemical.

in which animals (usually rats and dogs) are dosed daily. Initially, it begins at a therapeutic level and hence gradually increasing until the toxic effects start to be observable. Repeated dosing of the substance is done but at a lower dosage than that used in acute studies to calculate the safe dosage limit. The behavioral, physiological, body weight, food consumption, and biochemical changes of the organism are monitored.[88,89]

In Vitro, In Vivo, and In Silico Toxicity Assessment. *In Vitro Assay.* In vitro toxicity assessment involves a set of techniques meant for the screening of potentially toxic substances. The study helps to calculate the dosage limits of exposure and the fate of the xenobiotic component exposed.[90] In this study, different cell lines are exposed to several potential toxic substances and left for incubation for definite time intervals. The proliferation and the cellular metabolism of these exposed cells are measured using different assays (MTT, WST-8 etc.).[91] The assays used for these screenings are discussed in the later section of this review. In vitro studies are highly recommended because the results are obtained very rapidly and involve cost-efficient techniques. Moreover, in vitro

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studies do not require the use of animals, which avoids any form of ethical issues. These studies aim to mimic cellular events inside the human body after being exposed to any toxic substance. However, it suffers several demerits; the mimicking of cellular events often does not correlate with the original physiological outcome (as studied in in vivo study).[37] The prediction of the fate of the xenobiotic compound and the physiological end points after their exposure is highly critical and still requires intensive studies.[92−95]

In Vivo Assay. In vivo toxicity assessment remains the prime standard to assess the toxicity of various substances.[96] In this technique, a small dosage of the toxic substance is administered inside the body of model animals such as mice.[97,98] The cellular uptake, distribution, metabolism, and the removal pathway can be studied in this technique. Even though this method involves a great extent of time and cost, the results obtained are more reliable than other methods. This makes this type of assay still the most acceptable approach to analyze and understand the toxicity of substances.[99−101]

In Silico Assay. The need for rapid and reliable toxicity analysis of varied materials has prompted researchers to look for other alternative methods. In silico method is one of the relatively novel approaches compared to the conventional in vitro and in vivo assessment techniques. It utilizes several theoretical models to predict the physicochemical properties of the molecules. This collective gathering of models is defined as quantitative structure−activity relationships. In an in silico prediction, the toxicity of any molecular compound is predicted using the available experimental data and further interpolating using mathematical models.[102−104]

In silico prediction is highly desirable because of its rapid and cost-efficient technique.[102] It also avoids any ethical conflicts, as there is no use of animals. However, it too suffers from its own set of challenges. Prediction of the dosage and the type of toxicity is highly acute, and defining the toxicological end points after the exposure requires additional experimental verification.[105−108]

OTHER PHYSIOLOGICAL IMPACTS DUE TO NANOMATERIAL INTERACTION

Reactive Oxygen Species (ROS) Generation and Oxidative Stress. The presence of ROS is significant in the human body for several metabolic functions, but variance in the measure of ROS in the cellular environment can cause modulation of several cellular events such as signal transduction and protein redox potential.[93,109] Therefore, ROS generation due to any xenobiotic component can induce different levels of toxicity inside the exposed cells. They originate on the nanoparticle surface due to their electronic properties or while interacting with the cellular environment.[110−112] Figure 10 provides a summarized outlook of different ROS generation.

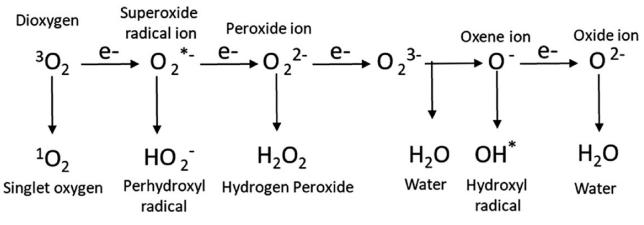

Figure 10. Generation of different ROS by the reduction of ground state oxygen. Adapted with permission from ref 113. Copyright 2004 Annual Reviews.[113]

Usually, in the case of metal oxides, when the conduction band of the metal oxides overlap with the cellular redox potential (−4.12 to −4.84 eV), it leads to the generation of potential ROS species and reduces the optimum levels of antioxidants present in the cell.[109] When the level of ROS exceeds the cell's ability to neutralize them, it leads to a state termed as oxidative stress. Oxidative stress enables the activation of several redox signaling pathways which involves MAPK (mitogen-activated protein kinases) cascades.[113] These protein cascades are responsible for various cellular processes such as proliferation and differentiation. These protein cascades help in the expression of proinflammatory cytokines, chemokines, and adhesion molecules. At lower oxidation stress levels (namely tier 1), the genetic antioxidant response element is activated, which results in the expression of antioxidant enzymes. Subsequently, the antioxidant response element activates the transcription factor Nrf2.[114,115] This transcription factor expresses for several antioxidants, cytoprotective and anti-inflammatory enzymes affecting the lungs which describe the stress mitigation observed for tier 2 levels.[116] The antioxidants are the proteins acting to remove the oxidative stress from the cell. There are two types of antioxidants: primary defense (superoxide dismutase, GSH, catalase, and thioredoxin reductase) and secondary defense (reduced glutathione).[117] The primary defense antioxidants react with the highly reactive superoxide to form less reactive hydrogen peroxide.[93] GSH is a nonprotein thiol which maintains the cellular redox levels. GSH exists in two forms: glutathione disulfide (GSSG), the oxidized form, and the reduced GSH. The balance of both these forms is done by the action of GSH reductase. It is one of the primary antioxidants that reduces the release of cytokines and chemokines by decreasing the activation of NF-κB. Variation in the GSH or the GSSG levels due to oxidative stress in response to the inflammatory mediators can induce the flow of several enzymes related to redox system such as GCLC, GSH peroxidase, MnSOD, etc. The decrease in the levels of GSH results in increased membrane permeability and activation of NF-κB.[118−121] Finally, when the oxidative stress levels overwhelm (tier 3), then the mitochondrial permeability is disrupted, and it meddles the electron transport. This finally leads to cellular apoptosis and necrosis.[122,123] The scheme in Figure 11 provides a summarized overview of the impact of oxidative stress.

Besides all the negative aspects of oxidative stress and the resultant cell death, researchers have been utilizing this very feature for cancer therapy and drug delivery. Li et al. in a recent report utilized a lipid enveloped functionalized drug delivery vehicle to deliver siRNA (small interference ribonucleic acid). The formation of new blood vessels (angiogenesis) and lymph vessels (lymphangiogenesis) promotes the growth of tumor and cancerous cells. Hence, inhibiting the growth factor signals, facilitating this formation by the siRNA can effectively contribute in controlling the cancerous cell growth. Hence, nanocarriers responsive to reactive oxygen species developed in the cytosolic environment help in the timely release and effective delivery of the siRNA (Figure 12).[124]

Inflammation. It is one of the important toxicological outcomes induced as a defensive action against any infection or xenobiotic material. The effect of the inflammation depends on the external stimuli and on the nature of the affected tissue. An inflammatory pathway (Figure 13) consists of inducers, sensors, mediators, and effectors, and inflammation can take place in any tissue. The xenobiotic components are basically the inducers which are detected by the inflammatory cells

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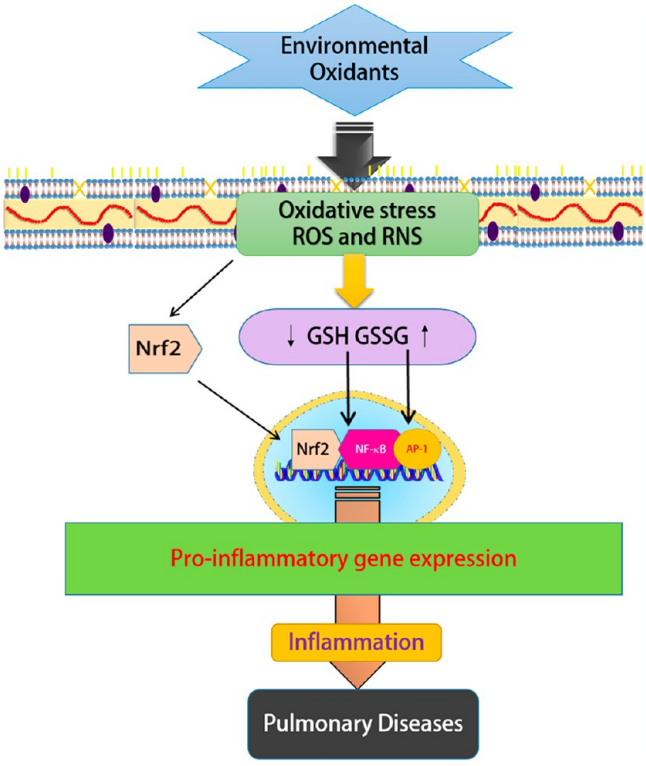

Figure 11. Oxidative stress results in the variation of the redox levels and results into several pulmonary diseases. The modulation of GSH/GSSS levels results in the activation of proinflammatory genes such as NF-kB. Various thiol compounds such as *N*-acetyl-L-cysteine (NAC) and *N*-acystelyn (NAL) supply cysteine for biosynthesis. Adapted with permission from ref 118. Copyright 2009 Elsevier.[118]

which behave as potential sensors against them. The cellular uptake of the material leads to the production of soluble factors (mediators) such as chemokines, cytokines, vasoactive amines, eicosanoids, and products of proteolytic cascades. The mediators initiate the flow of plasma protein and leukocytes (neutrophils) along the venules present at the site of exposure (infected cells). The neutrophils get activated on interaction with the infected cells and initiate the cleansing task by the release of ROS, reactive nitrogen species, proteinase 3, cathepsin G, and elastase. However, these cleansers cannot discriminate between the infected (exposed) cells and the healthy cells. This leads to a collateral damage, but the repair phase is initiated after the inflammatory response.[118,125,126]

Nonoxidant Routes to Cellular Injury. There are several nonoxidant methods to cause cellular injury. Excluding the surface energy states of the nanoparticles, which regularly interact with the cellular environment to produce ROS, nanoparticle dissolution is one of the significant forms of inducing toxicity in cells. The particle dissolution is a thermodynamic property and requires a negative surface free energy. It is an energetically favored reaction between a particle and solvent. The NPs surface area, surface energy, surface morphology, aggregation status, concentration, and adsorbing species have a major influence on its solubility.[127,128] Nanoparticles such as zinc oxide (ZnO) and ferrous oxide (FeO) induce more toxicity than the less soluble metal oxides such as ceria (CeO_2) and titania (TiO_2).[129] Similarly, Latiff et al. observed the difference in the cytotoxicity of gallium selenide (GaSe) compared to the other commonly known transition metal dichalcogenides (TMD) such as WS_2, WSe_2, and MoS_2 because of the high solubility of the Ga ions in the cellular environment.[130] The influence of particle dissolution for cytotoxicity initiation by metal oxides was explained by Limbach et al. In this study, they introduced the concept of a Trojan horse mechanism to illustrate the toxic potential of several transition metal oxides. The study revealed that the cells exhibited 25 times greater oxidative stress when subjected to metal oxides compared to their metal solutions.[131] The cellular membrane prevents the entry of the metal ions; therefore, they exhibit a controlled level of oxidative stress. While the metal oxide nanoparticles can easily enter the cytosol, at a defined pH (as suitable for their dissolution), they start to leach the metal ions, which further initiates potential cytotoxicity.

Nanoparticles inside the cellular environment interact with several biomolecules. These biological molecules compete to

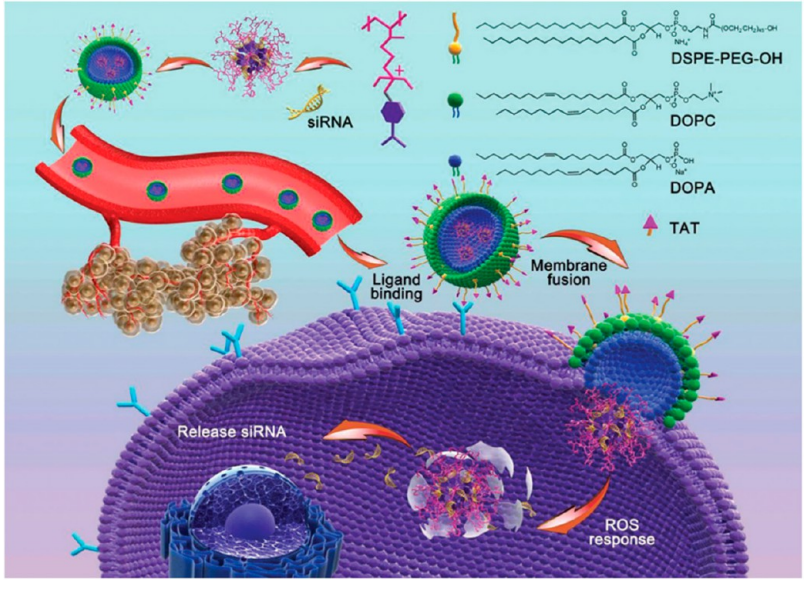

Figure 12. Schematic illustration of a lipid-coated ROS-responsive polymer for siRNA delivery. Reproduced with permission from ref 124. Copyright 2018 The Royal Society of Chemistry.[124]

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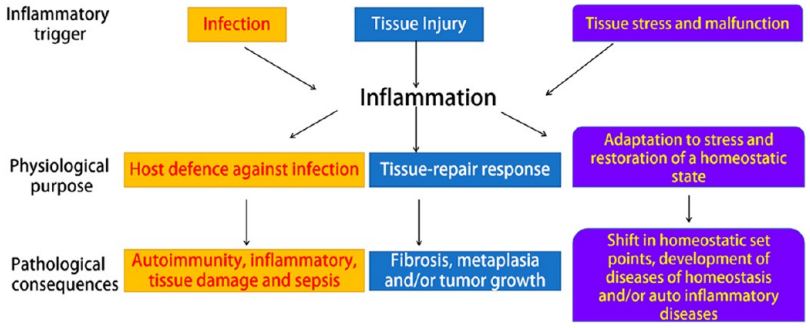

Figure 13. Inflammatory pathway. Adapted with permission from ref 126. Copyright 2008 Macmillan Publishers Limited.[126]

get adsorbed on the surface of the nanoparticles. Initially, the protein molecules get adsorbed on the surface and form a nanoparticle−protein complex known as the corona (Figure 14).[132,133]

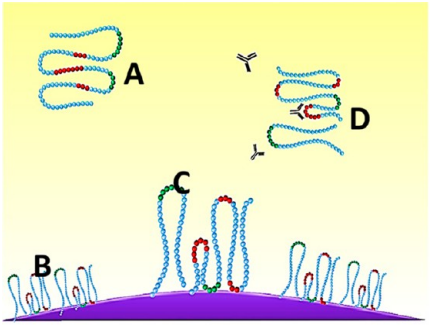

Figure 14. Schematic representation of the formation of nanoparticle−protein complex: (A) protein, (B) NP−protein interaction, (C) NP−protein complex; the surface of the NP induces conformational changes in the protein molecule. (D) The following interaction induces toxicological changes in the cellular environment. Reproduced with permission from ref 135. Copyright 2013 Shruti R. Saptarshi, Albert Duschl, and Andreas L. Lopata.[135]

The physicochemical characteristics of the nanomaterial (size, shape, composition, surface functional groups, and surface charges), the features of the physiological surroundings (blood, interstitial fluid, cell cytoplasm, etc.), and the duration of exposure are the most significant factors influencing the structure and composition of the corona. These protein coronas initiate perturbed biological functions due to the variation in the structure of the proteins and the local high concentration. These protein coronas also influence the cellular uptake, inflammation, accumulation, and degradation of NPs.[132,134−137]

EFFECT OF DIFFERENT PHYSICOCHEMICAL PROPERTIES IN THE CYTOTOXICITY ASSESSMENT OF THE NANOMATERIALS

The physicochemical properties of the nanomaterials are a very important factor to be considered (Figure 15).[138,139] There are numerous materials which are nontoxic in bulk but exhibit intense toxicity as their size reduces. Apart from the dimension of the material, the chemical composition, especially its surface properties and surface area, also plays a significant role in inducing toxicity.[140−142] There are multiple factors contributing to the toxicity of any material, but the present section discusses a few of the principal ones.

Size. Particle size is one of the prime physicochemical properties which affect the toxicity of materials.[143] The materials induce toxicity, as they are exposed to the cellular environment

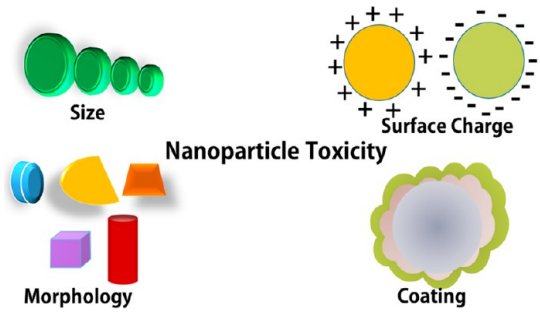

Figure 15. Physiochemical factors affecting cytotoxicity.

or ingested using different intake pathways (inhalation, absorption through skin, etc.). The intake of these materials occurs so easily because of their comparable size with the biomacromolecules. The surface area of the materials increases with the decrease in size, which provides an increased surface for interaction in the cellular environment. There exist several toxicity studies illustrating the importance of size in toxicity and verifying that decreased size is the prime cause of toxicity induction. The nanomaterials upon entry in the cellular environment interact with the proteins present in the cytosolic fluid and form a nanoparticle−protein complex. The NP−protein complex induces physiological changes inside the cellular environment. Moreover, the decreased size also increases the production of ROS. The decreased NP size has increased surface area, which renders increased sites for ROS production. However, the toxicity influence by ROS and the NP−protein complex is explained in the latter half of this review.[144,145] Park et al. studied the effect of Ag particle size on L929 fibroblasts and mouse peritoneal macrophage cell lines (RAW264.7). The silver (Ag) NPs exhibited cytotoxicity, inflammation, genotoxicity, and developmental toxicity. The smallest NP (20 nm) showed the highest toxicity compared to the other larger NPs. The Ag ions were more toxic compared to the NPs in the macrophage cells, not in the fibroblasts.[146] Chen et al. studied the toxic influence of titania particles on different cell lines: Smulow−Glickman (S−G) gingival epithelial cells, oral mucosa fibroblasts (OMFs), normal human bronchial epithelial cells (BEAS), and lung fibroblasts (WI-38). The study aimed to establish a correlation between the morphology, size, and the cell lines on the cytotoxicity. The cells internalized the NPs very easily but remained aggregated in the cytoplasm. The spherical NPs were internalized very easily compared to rod shaped large particles. The toxicity of the NPs varied significantly along with the cell type.[147] In another analysis, authors studied the influence of surface functionalization and the size of the titania nanoparticles on internalization and cytotoxicity. The study

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found that the NPs less than 100 nm were internalized easily inside the cells. The internalized NPs were bounded inside small vesicles present in the cytoplasm. The presence of N^+ in the phospholipid layer results in the prime interaction with the functionalized titania NPs.[148] Studies illustrating the oligodynamic effect of Ag NPs have been demonstrated in the past few years. Even a low dosage of nanoparticles and the interaction of the leached ions destroy the cell membrane and contribute to the bactericidal behavior.[149,150] Agnihotri et al. studied the oligodynamic effect of Ag NPs, which were amine functionalized on an immobilized silica glass surface using 3-(2-aminoethylaminopropyl)trimethoxysilane as cross-linkers. The glass substrate showed that cell proliferation ceased within 2 h, and the substrate was reused several times.[151] In contrast, Dupont et al. discusses the genetic modification in bacterial protein structures, as a resistive mechanism to metal NPs. The abundant availability of these metal nanoparticles poses a challenge for bacterial proliferation but at the same time offers sufficient room for them to develop resistive mechanism against these metal NPs.[152] This has eventually renewed the interest to provide a new understanding to the existing discussion.

In the case of quantum dots, the route of preparation influences its size and it further induces the cytotoxicity of the nanomaterial.[153,154] Quantum dots prepared using an organic synthetic route has an organic ligand such as trioctylphospine. These ligands are hydrophobic in nature and require post-synthetic treatment to turn them hydrophilic, and hence, the hydrodynamic radius of these particles increases compared to the quantum dots prepared in aqueous solution.[155] Thus, a change in the size of the nanomaterials can influence the outcome of the cellular viability, which can be effectively used for therapeutic purposes such as drug delivery, imaging, and cancer treatment in vivo.[156−158] Varying the size of a single drug can cause the destruction of the cells and at the same time help to serve drugs to their prescribed destination.[159,160] In one study, the authors evaluated the quantum dots of different sizes and coating properties for their ability to penetrate skin and their further localization and toxicity estimation. The study inferred that the quantum dots of different physicochemical natures had the ability to penetrate the stratum corenum barrier and remain localized for 8 h in the epidermal layers. Such impregnation results in localized inflammation and cytotoxicity.[161]

Morphology. The morphology of the particle influences significantly in the cellular uptake mechanism.[162] The role of particle shape and aspect ratio is described in various toxicity studies.[163,164] Nanomaterials have different shapes such as spheres, filaments, planar, tubes, etc.[93] The shape of the particle plays a key role in inducing toxicity only at the time of ingestion inside the cellular environment. The endocytosis of NMs is dependent on size and concentration (as discussed in an earlier section).[165] Doshi et al. studied the influence of particle shape on cell membrane interaction. They observed that the needle-shaped particles induced disruptions of cell membrane during their uptake.[166] Similarly, other studies have mentioned that the intake of NMs of high aspect ratio leads to the formation of pores in the cell membrane. This causes misbalance of the ionic concentration inside and outside the cell. Moreover, the NPs often lead to aggregation after its up-taken inside the cell. It leads to a significant variation in the outcome of the size dependent study and might influence the overall result.[167]

Several other studies such as Chithrani et al. have demonstrated the influence of both size and shape of gold nanoparticles on the uptake into mammalian cells. It was observed that the cellular uptake increased up to 500% on just tailoring the morphology of gold (Au) NPs (from rod-shaped to spherical).[168] Thus, optimizing the shape dependent attributes of nanomaterials can help in tuning intracellular delivery rates and much more.[169] Pasqua et al. assessed the toxicity of several silica NPs in this study using human neuroblastoma (SK−N−SH) cells (dosage up to 800 μg/mL and exposed for 48 h). The mesoporous silica NPs were found to be the most toxic among them, and the least was observed for the spherical NPs. The presence of functional groups in the mesoporous silica and the high surface area rendered to be the prime cause of high levels of toxicity.[170] In a more recent approach, Vicente et al. studied the drug delivery efficiency of silica NPs and studied the toxicological profile of the nanoparticles of different sizes in two different cell lines (human keratinocytes (K17) and human dermal fibroblasts (HDF)). The toxicity of these NPs was dependent more on the cell type and the mode of internalization. Phagocytosis was the internalization mechanism for K17, and caveolae-mediated endocytosis for HDF.[171] The size of the particles was secondary and relevant for particles of smaller size (<20 nm). Hashimoto et al. investigated the toxicity influence of the morphology of alumina nanoparticles and nanowires. The nanomaterials were exposed to 2 different cell lines (fibroblasts L929 and macrophage RAW264) for 24 h. The study revealed that the nanoparticles were cytotoxic and as well as genotoxic in nature, while the nanowires did not show any toxicity. The localization of these nanomaterials was studied using scanning electron microscopy (SEM) and transmission electron microscopy (TEM). The nanoparticles entered the nuclei and the vesicles and caused nuclear damage, while the nanowires were retained only on the surface of the cells. The increased surface area promoted the increased levels of toxicity.[172] Similarly, Ji et al. studied the influence of the aspect ratio on the toxicity effect of ceria nanorods and nanowires by exposing human monomyelocytic leukemia cell lines (THP-1) for 24 h and later studying the influence using LDH assay. The results showed that the short ceria nanorods were not at all toxic, while the nanorods of intermediary aspect ratios did not cause obvious cell death but did induce IL-1β production. The rods with the highest aspect ratio exhibited maximum toxicity and released the maximum of IL-1β.[173] In a much more recent effort, Maysinger et al. studied the influence of gold nanourchins (nanoparticles with irregular morphology) and their effect on glioblastoma cells. The results showed that the viability and the morphology of the cells remained unaffected by gold NPs functionalized by polyethylene glycol (PEG), while addition of celastrol caused significant attribution. On the other hand, cetyltrimethylammonium bromide (CTAB) modified gold NPs adversely affected the nuclear lamina, microtubules, and filamentous actin.[174]

Surface Charge. Toxicity of a material is induced because of cellular interaction with the surface of the nanomaterials. Hence, the surface properties are the key factors to induce toxicity. The NMs are ingested inside a cell by crossing the lipid bilayer membrane. The overall charge of this membrane is negative. Hence, if the NMs are positively charged or neutral in nature, they can be easily ingested and get simply bound to the cell membrane using electrostatic interaction,[175] whereas negatively charged particles are bound less efficiently. The toxicity assay of several materials was performed in different studies, where the effect of surface charge and functionalization is studied. Magrez et al. observed significant changes in toxicity after surface functionalization of carbon nanotube (CNT) with

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acid treatment. An acid wash converted the less toxic CNTs into highly toxic entities. The acid wash of CNTs added negatively charged functional groups such as the hydroxyl group (−OH) and carboxylic acid (−COOH), which contributed to the variation in toxicity.[176] Cho et al. studied the cellular adsorption of Au NPs on the cell surface. The kinetics of Au internalization was studied, and it was observed that the cationic Au NPs had an internalization rate five times higher than that of the anionic Au NPs. The endocytic routes of these NPs are also varied. The authors believed that the cationic Au NPs diffused directly inside the cells by disrupting the cell membrane.[177] This postulation was further verified in several other publications.[175,178] Interaction of surface functionalized NPs leads to the damage of the integrity of cell membrane and results in pore generation. Other than the surface charge, the ligand interaction (after surface functionalization) also costs in variation in toxicity assessment.[179,180] Peetla et al. studied the effect of the molecular structure of cationic ligand on the cellular uptake through a model membrane. It was observed that a single chained and double-chained cationic ligand showed different mechanisms of interaction.[181] In another study, the influence of surface modified Au NPs in the cellular uptake is studied. Four NPs had very similar physical characteristics except for the composition and structure of the ligand shell. The NPs showed difference in the cellular intake; two of them entered the cytosolic environment without disrupting the bilayer membrane, but the other two were trapped inside the endosomes.[182]

Coating. The surface of any material is the initial route of interaction with the cellular environment. Hence, decreased size, surface charge, the presence of ligand, and its orientation are several such factors which contribute to the toxicity of any substance. There are several instances when the toxic metal ions get solubilized in the cellular environment and induce acute levels of toxicity.[128,131] Therefore, studies have been performed to develop coatings around these metal oxides, and such credible toxicants to prevent instances of leaching. These coatings can be three common types, as discussed by Richards et al.:[183] (1) covalent surface coating, where the coating is adhered around the molecule by covalent bonding; (2) electrostatic surface coating, the attraction of opposite charges between the surface of the coating and the molecule keeps them adhered; and (3) atomic layer deposition (ALD) (Figure 16), a coating

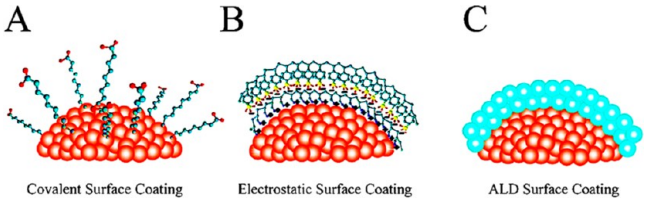

Figure 16. (A) Covalent surface coating, (B) electrostatic surface coating, and (C) ALD coating. Reproduced with permission from ref 183. Copyright 2012 The Royal Society of Chemistry.[183]

developed around the molecule using ALD which results in the formation of chemical bonds between the coating material and the molecule. These coatings decrease the toxicity levels, stabilize the particles, prevent them from agglomeration, and simultaneously increase the cellular uptake.

The use of coatings around potentially toxic molecules can help in modulating for several applications such as drug delivery, imaging, and cancer treatment.[184] The use of chemotherapeutic drugs for cancer treatments often lead in the death of healthy cells and induce toxicity (Figure 17). Therefore, studies discussing selective targeting of the cancerous cells using "nanocarriers" coated with molecules, which bind with the overexpressed antigens present in the target cells, are highly desirable.[185] In another study, the polymeric nanoparticle poly(lactic-*co*-glycolic acid) (PLGA) has additionally been utilized as a nanocarrier for drug delivery (loperamide) across the blood-brain barrier due to its biocompatibility and biodegradability, thus warranting safe treatment.[186] Similarly chitosan-based micro/nanoparticulate have been assessed for drug delivery applications. Their ability to facilitate both protein and drug conjugation and enhanced permeability with retention effects but reduced immunogenicity makes them perfect candidates.[187] Copper nanoparticles exhibit a high inflammatory response; surface modification with chitosan reduced the ROS production, but the inflammatory response enhanced again when they were administered through lungs.[188] Another study showed that the coating of chitosan around Fe_2O_3 nanoparticles reduced cellular damage and lessened the ROS production, thereby affecting the overall toxic influence of the nanoparticles.[189] Polymer coatings of PEG around superparamagnetic iron oxide nanoparticles (SPIONS) have lessened the toxicity effects of the nanoparticles by blocking the interaction of the nanoparticles with the ROS generated in the cytosolic environment, thereby giving the cell's antioxidant mechanism enough time to neutralize the ROS generated prior to their becoming toxic.[190]

Yin et al. studied the influence of particle size and surface coating on the cytotoxicity of nickel ferrite. The authors compared the cytotoxicity of nickel ferrite NPs and the influence of surface coating. Uncoated NPs (with oleic acid) showed dose independent toxicity, and moreover, the difference in particle size did not render any influence over the toxicity, while the oleic acid coated NPs showed dose dependent toxicity. The nature of the coating changed with the number of layers. Single coating turned the surface hydrophobic and double coating as hydrophilic. The hydrophobic coating imposed high levels of toxicity and vice versa for hydrophilic coatings.[191] Alkilany et al. inspected the influence of the aspect ratio of gold NPs in the cellular uptake and cytotoxicity. The Au NPs were coated with two different polymers, CTAB and poly(acrylic acid) (PAA). The CTAB coated Au NPs showed low cell viability irrespective of their aspect ratio. Conversely, the PAA coated Au NPs showed the reverse attribute. The free CTAB molecules were the prime reason for toxicity irrespective of their surface charge.[192] Similarly, Au NPs coated with PEG and a nuclear localization signal (NLS) peptide were capable to evade the endosome and pierce the nucleus of cancer cells to prompt DNA destruction.[193] In another study, the influence of the surface coatings on the cytotoxicity of several Ag NPs was evaluated using cell lines of mouse macrophage (RAW-264.7) and lung epithelial (C-10). The nature of the coating played a significant role in various levels of toxicity. Poly(diallyldimethylammonium)-coated Ag NPs showed the highest toxicity, and the uncoated Ag NPs showed the least. Additionally, the lung cells showed more restraint to the intake of the Ag NPs compared to the macrophages.[194] Similarly, in another analysis, authors studied the effect of size and two distinct types of coated Ag NPs (polyvinylpyrollidone (PVP) and citrate) in BEAS cells. Neither the cellular uptake nor the toxicity showed any significant differences in the coated Ag NPs. However, the smallest Ag NPs (10 nm) showed the highest toxicity, which is attributed to the release

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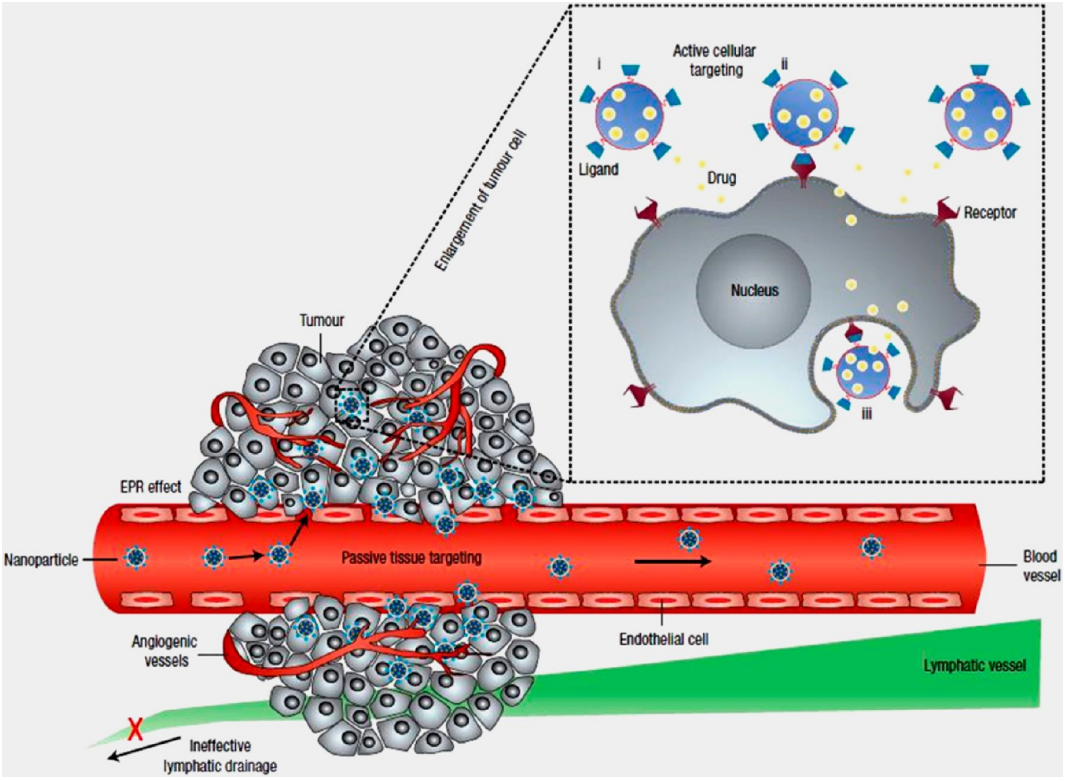

Figure 17. Schematic representation of different mechanisms by which nanocarriers can deliver drugs to tumors. Reproduced with permission from ref 185. Copyright 2007 Nature Publishing Group.[185]

of more Ag ions (Trojan horse effect).[195] Dong et al. studied the influence of Cu doped Ag_2S nanoparticles coated with PVP. The as-prepared nanoparticle composite showed high photothermal conversion efficiency and was further utilized for in vivo photoacoustic imaging guided photothermal therapy. The biocompatibility of the nanoparticles was assessed using MTT analysis using 4T1 murine breast tumor cells.[196]

PATHWAYS FOR CELLULAR UPTAKE OF NANOPARTICLES, TRANSLOCATION, AND THEIR OUTCOMES

There are several features influencing the nanomaterials adsorption, distribution, metabolism, and excretion. Nanomaterials are extremely small, which helps them in their easy uptake inside the body and enables them to overcome different biological blockades. The large surface area, surface properties, and numerous such features affect the toxicity of the material. Among all of them, the route of uptake plays a small role in inducing toxicity. However, it is critical to understand how these nanomaterials are ingested inside the cell and their fate after internalization.[197] The literature provides several uptake mechanisms, but the current review discusses the most common form of internalization (Figure 18).[7]

Endocytosis. Endocytosis is a process utilized by the cells to ingest extracellular components. This form of active transport encloses the object present outside the cell, within inward folding of the plasma membrane, which further gets pinched off from the surface to form intracellular vesicles.[198] The ingested foreign object/materials are delivered to lysosomes for further degradation.[199] There exist several endocytic pathways utilized by the cells to internalize several types of particles, which are phagocytosis, pinocytosis, clathrin-mediated endocytosis, and caveolae-mediated endocytosis.[79,200,201]

Clathrin-Mediated Endocytosis. A receptor-mediated endocytosis process in which materials of size usually <100 nm are taken into the cell from the surface using clathrin-coated vesicles. In this process, the plasma membrane undergoes inward budding and form vesicles. These vesicles are coated with different protein receptors enabled to internalize the specific molecule.[202] In this energy, dependent process the clathrin do not interact directly with the membrane or the cargo (ingested particles) receptors. It relies completely on the protein receptors present on the walls of the vesicles and the accessory proteins. These accessory proteins are the cytoplasmic proteins which are further reused for another endocytosis cycle. The ingested particles undergo sorting in the endosomes and are further sent to the surface or delivered to other mature endosomes such as lysosomes.[203] The clathrin-mediated endocytosis is responsible for several functions such as uptake of nutrients, activation of signaling pathways, regulating the surface expression of proteins, retrieving proteins deposited after vesicle fusion, etc.[203−207]

Caveolae/Raft-Dependent Endocytosis. The caveolae-dependent endocytosis is one of the potential uptake methods of nanoparticles of size <200 nm. It is a clathrin-independent endocytosis method which is a combination of pinocytosis and endocytosis mediated by caveolae and glycolipid rafts. Caveolae are cholesterol and sphingolipid-rich invaginations of the plasma membrane, and glycolipid rafts are membrane fractions rich in cholesterol and sphingolipids. These invaginated domains of the plasma membrane are distinguished by the presence of the integral membrane protein caveolin. This method of entry can prevent the cargo (ingested particles) from digestion in lysosomes. Thus, this receptor-independent endocytosis can be used efficiently for drug or DNA delivery applications.[208−211] In fact, Rejman et al. studied the importance

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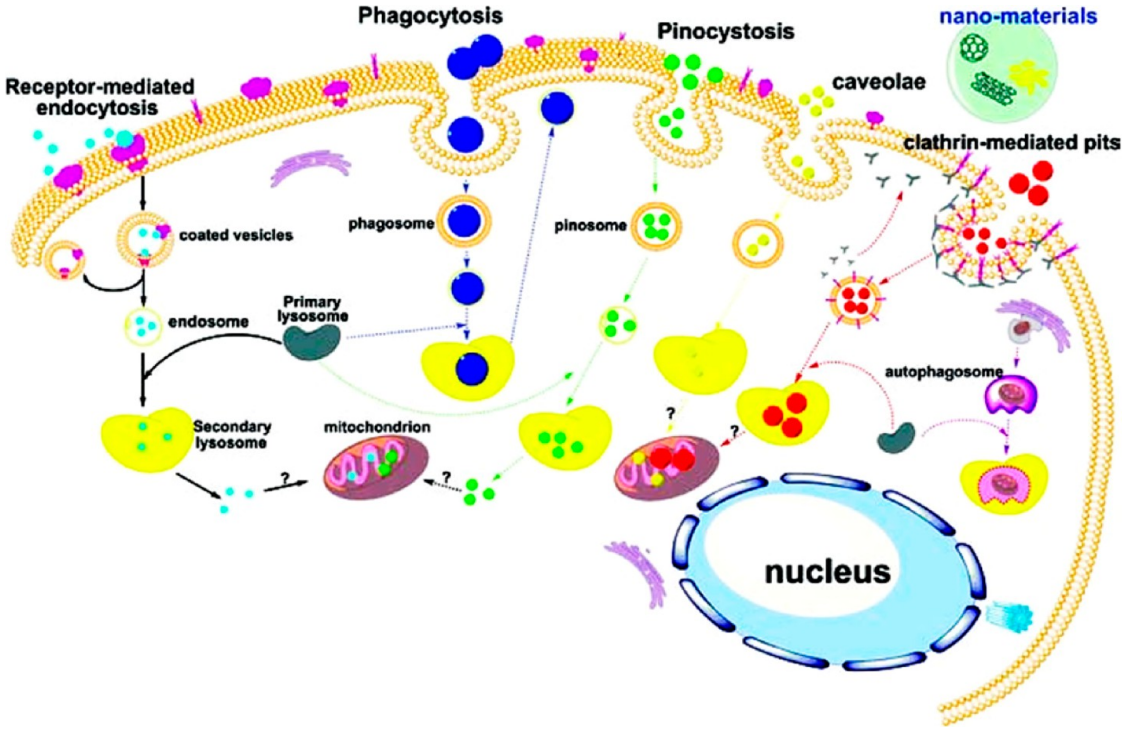

Figure 18. Schematic of different cell uptake pathways. Reproduced with permission from ref 221. Copyright 2011 John Wiley and Sons.[221]

of the size of the particles in different endocytosis process.[212] This study revealed that the size of the particles determines its entry portal. Hence, a proper understanding of other kinetic parameters of internalization can improve the drug delivery efficiency.

Phagocytosis. Phagocytosis is an endocytic process of internalizing particles >500 nm. Mammalian cells such as mononuclear phagocytes, macrophages, and neutrophils utilize phagocytosis to remove infectious particles or cellular debris. These specialized cells have evolved their functioning ability and participate in the uptake of nutrients, development and remodelling of tissues, immune response, and inflammation. The internalization process is initiated by the interaction of the receptors on the cells with the ligands on the particles.[213,214] The presence of specialized molecules such as antibodies labeled on the surface of the ingested particle can speed up the phagocytosis process; this process of labeling is termed opsonization. Further, it results in polymerization of actin and leads to the internalization of the particles via an actin-based mechanism. The internalized cell inside the phagocytic cells combines with the lysosomes (contains digesting enzymes) to form phagolysosomes. This fusion takes a longer time, which depends upon the kind of interaction between the surface of the particle and the phagosome membrane.[215,216] The ingested particles undergo complete breakdown inside the compartment using different proteases and nicotinamide adenine dinucleotide phosphate (NADPH) oxidases. Further, the residue after the breakdown is removed out from the cell via exocytosis.[7] The phagocytosis process is very complex in nature because of the presence of different kinds of receptors enabled to initiate the phagocytosis process and the difference in fate. The main challenging task of these cells is to differentiate between potential pathogens and self. However, this task is accomplished by several phagocytic receptors that have acquired the ability to discriminate between them.[217,218]

Pinocytosis/Macropinocytosis. Pinocytosis is an endocytic uptake process occurring in all cell types, which internalizes particles from few to several 100 nm. In this process, it leads to the formation of membrane-based vesicles from the cell surface that uptakes fluid and the solute from the exterior environment. These ingested pinocytic vesicles fuse along with the lysosome.[219,220]

POSSIBLE HUMAN EXPOSURE TO NANOMATERIALS

Human exposure to nanomaterials can happen in numerous routes and at various phases of nanomaterial synthesis. The human body gives a few interfaces such as skin, gastrointestinal tract, and respiratory tract for transporting nanomaterials (Figure 19).[7]

Skin. Skin is one of the largest organs and the primary shield of our entire body and is the easiest route for entrance. The epidermis of our skin denies entry of micrometer-sized particles, but this barrier is unproductive for particles in the nanodimension. Dermal exposure is unavoidable with drug treatments and application of creams (sunscreens and others). The epidermal entry for nanoparticles is governed by varied factors such as the medium of exposure, pH of the medium, temperature, etc.[222−224] Underneath the dermal layer is rich with blood and macrophages, lymph vessels, dendritic cells, and nerve endings. Hence, the particles that get absorbed beneath the different layers of skin get readily transported within different circulatory systems.[7,225]

Respiratory Tract. Nanoparticles dispersed in the air such as carbon and asbestos can enter the body through our respiratory tracts. After inhalation, the nanoparticles get deposited all over the respiratory tract, starting from nose to lungs.[7] Their small aspect empowers them to surf over the alveolar region of the lungs and enter the blood and the lymph system. The capillary tubes present in the alveoli provide quick diffusion,

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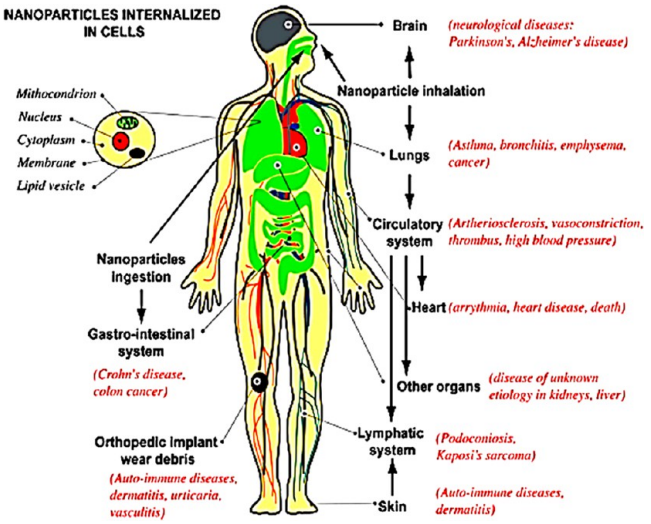

Figure 19. Schematic illustration of a human body, indicating the exposure routes of various nanoparticles and their adverse health effects. Reproduced with permission from ref 7. Copyright 2007 American Vacuum Society.[7]

but the intake and absorption of the toxic particulates depends on the morphology, concentration, etc. The large surface area of the lungs helps it to serve as a site of absorption and desorption of the toxic elements. The xenobiotic component might undergo biotransformation in the lung cells and end up being desorbed from the body through excretion. Thus, the concentration and morphology govern the intake of the toxicants through the respiratory tract. The respiratory tract usually provides the pathway to enter the gastrointestinal tract or more often they serve their entrance via food, water, cosmetics, drugs, etc.[7] The particles less than 10 mm end up being adsorbed inside the lungs and might get translocated to different parts of the body such as a kidney. These materials are further removed partially or completely from the body through the mucociliary escalator and by phagocytosis.[222−224] Insoluble particles deposited in lungs could initiate various toxicological responses on the site. The translocation of smaller NPs is easier compared to the bigger ones, and they are removed faster from the lungs compared to the bigger particles. They may get deposited in the lungs, and once entered into the respiratory epithelium, they can remain there for years and can possibly enter the lymphatic system and circulatory system. They may further channel to other parts of the body such as the liver, spleen, kidneys, etc.[7] Nanomaterials such as carbon black, asbestos, multiwalled carbon nanotubes (MWCNT), etc. are some of the insoluble nanomaterials that get deposited on the lung surface. Methods like mucociliary escalator and alveolar macrophage phagocytosis are used to evade the toxicity imposed by these insoluble nanoparticles. However, the lung defense mechanism starts to act aggressively once these methods cannot control the spread of toxicity, and this eventually causes damage to the lung tissue.[7,226,227]

Gastrointestinal Tract. Potential toxicity can be directly ingesting contaminated foods or intaking toxic drinks. The GI tract provides enough opportunity for the toxicants to get absorbed into the body and can be easily translocated into the circulatory system. The epithelial cells inside the stomach are different from the other parts of the body. These cells are meant for absorption, and thus, any toxic element can be readily absorbed. However, there are multiple factors which govern this rate of absorption. The physicochemical properties of the toxicants such as the size, shape, concentration or dosage, pH of the medium, etc. are several key apprehensions.[222−224] Szentkuti et al. studied the relevance of size and electric charge in the penetration of nanomaterials. The study revealed that positively charged nanoparticles get trapped in the negatively charged mucus, and the negatively charged particles are easily ingested inside the mucus layer. Again, on the other hand, the size of the particles was found to be a necessary governing factor. The rate of ingestion was observed to be proportional to the diameter of the particles; the larger the diameter, the more time it required to complete the ingestion process.[228,229]

A summary of the adverse health effects and the possible uptake and translocation pathways of the nanomaterials are illustrated in Figure 20.[7,222]

Blood Brain Barrier (BBB). Until now, the review has been based on the use of NPs and their possible routes of toxic influence inside our body. The use of nanoparticles and their ability to initiate a ROS mechanism to curb toxicity is widely used for targeted therapeutic applications.[230] In such a matter, neurodegenerative diseases are one of the exciting sites of current research.[231] The need for drugs that can penetrate the BBB and effectively deliver drugs to the affected cells is critical. The central nervous system (CNS) is an important interface that controls the activities of the body. The BBB is the semipermeable membrane, which separates the transmission of the circulatory blood into the brain and cerebrospinal fluid.[232] Hence, this semipermeable membrane regulates the movement of ions, molecules, and cells between the blood and brain,[233] thereby enabling the CNS for proper neural function and prevents the pathogens to cause any neural disorder. The barrier is composed of endothelial cells that form the walls of the blood vessels. The physical transport and the metabolic properties of the endothelial cells are regulated by the interaction with different neural, immune, and vascular cells. Therefore, understanding the behavior of these cells around the BBB can help to attain answers to several neural disorders.

The use of nanoparticles is a potential answer in this avid search for the perfect drug carrier. Therapeutic agents made from nanoparticles have shown their ability to cross the BBB and targeting specific sites. Thus, understanding the pathological effects of the NPs around BBB (including its toxic influence) has commenced to be an imperative task. Gold, silica, and several other nanoparticles such as CNT and fullerenes are studied for their drug delivery application. As explained in a recent review by Saraiva et al., NPs classified as natural or synthetic with variable sizes get drug delivered using distinct techniques. The shape, size, and the surface charge definitely plays a crucial factor in determining the intake of these particles and channelling through the BBB (as discussed in Effect of Different Physicochemical Properties in the Cytotoxicity Assessment of the Nanomaterials).[234] Functionalization of these nanoparticles also articulates in this process (Figure 21).[235] The tight endothelium tissues of the BBB make the channelling of larger nanoparticles impossible.[7] Apart from the desired features of nanoparticles, the effect of nanoparticles and their impact on neurotoxicity is poorly understood. The overwhelming use of engineered nanomaterials in everyday products affects the toxicity and to a certain extent our functioning of BBB. Limited studies detailing this impact are available. In one such study, the authors showed that carboxylated polystyrene NPs (100 nm, 100 μg/mL, 24 h) exhibited reduced toxicity on hCMEC/D3 endothelial cells. The study demonstrated reduced levels of

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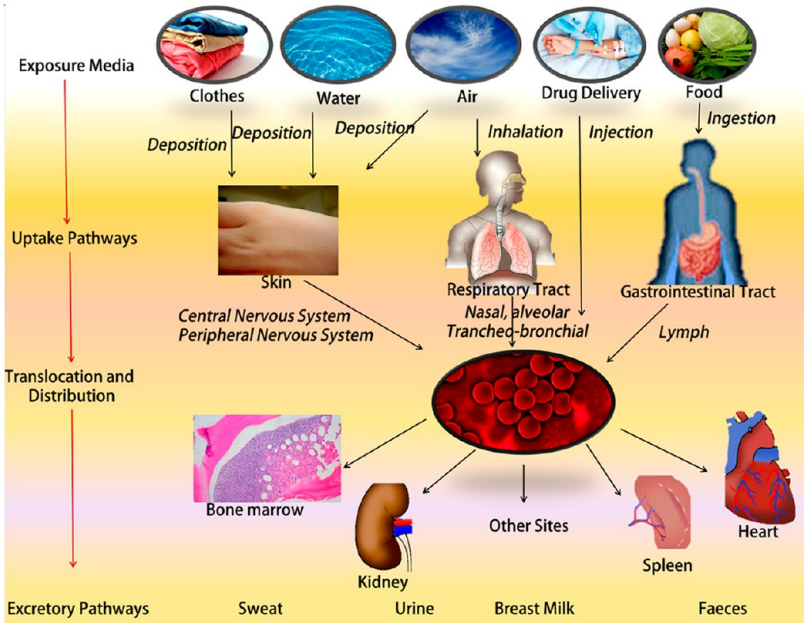

Figure 20. Summary of different uptake pathways along with their translocation routes. Adapted with permission from ref 222. Copyright 2005 Günter Oberdörster, Andrew Maynard, Ken Donaldson, Vincent Castranova, Julie Fitzpatrick, Kevin Ausman, Janet Carter, Barbara Karn, Wolfgang Kreyling, David Lai, Stephen Olin, Nancy Monteiro-Riviere, David Warheit, Hong Yang, and A report from the ILSI Research Foundation/Risk Science Institute Nanomaterial Toxicity Screening Working Group.[222]

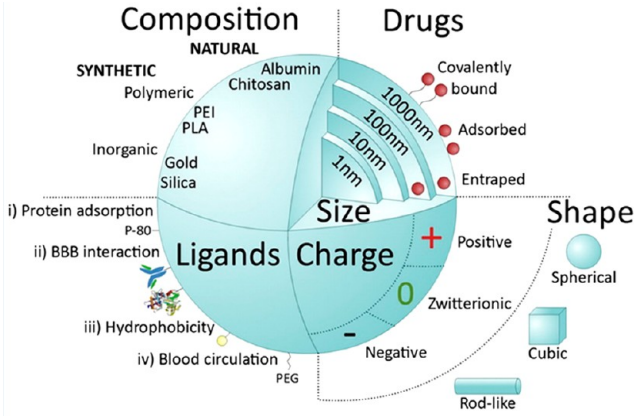

Figure 21. Schematic diagram illustrating the factors affecting the systemic delivery of nanoparticles in BBB. Reproduced with permission from ref 235. Copyright 2016 Elsevier.[235]

pro-inflammatory RANTES protein compared to normal condition, while on the other hand, on utilizing the same cell lines in the presence of astrocytes induced a significant release of pro-survival signaling. This illustrated the ability of nanoparticles in the modulation of the pro-inflammatory and pro-surviving proteins.[236] The same study showed that the carboxylated polystyrene NPs were found to be accumulated in the lysosomes without exhibiting any degradation.[236] Earlier reports suggested otherwise, but modifying the nanoparticles eventually illustrated improved results.[237] Gramowski et al. studied the effect of nanoparticles and their concentration over ROS formation. The study revealed that on exposing primary murine frontal cortical networks on microelectrode array neurochips for 24 h with TiO_2 exhibited concentration dependent ROS production, while carbon black NPs and Fe_2O_3 showed no change in the ROS production with an increase in the concentration levels.[238] In many instances the NPs interact with the cytoplasmic proteins of neurons; Xu et al. reported the disturbances of the synaptic structures and functions on exposing primary rat cortical neurons with Ag NPs (20 nm, up to 50 μg/mL). This exposure resulted in tampering of assembly and disassembly of cytoskeletal components in a dose dependent manner, finally resulting in the reduction of the synaptic clusters of the presynaptic vesicle protein synaptophysin and the postsynaptic receptor density protein.[239] Another study revealed the influence of NPs in their ability to interfere with gene expression. Exposure of Ag NPs in undifferentiated PC12 cells inhibited DNA synthesis and impaired the protein synthesis mechanism, while exposing the differentiated cell lines caused selective impairment of the neurite formation.[240]

Liver and Spleen. The liver is a complex organ and is anatomically and functionally assorted. It is the largest internal organ with different defense mechanisms against xenobiotics. The endothelial cells have large pores which make the entrance of the large NPs easier.[7] The easiest way of accumulation is intestinal absorption and further translocation to liver and spleen before entering the kidneys and circulatory system. Studies illustrate the deposition of carbon particles inside the livers of coal miners compared to those of normal patients.[222] Similarly, another evaluation suggests the deposition of wear particles to the liver and spleen of patients with hip or knee replacement.[241] Another study revealed the deposition of debris of dental porcelain bridges by intestinal absorption. This accumulation further resulted in acute renal failure, irregular bile flow, fever, etc.[242] The NPs are usually cleared from the liver by the biliary secretion into the small intestine; therefore, the biliary system may get exposed as well. In vivo results illustrate hepatocellular injury by a range of different mechanisms such as cytochrome P450 activation, alcohol dehydrogenase activation, membrane lipid peroxidation, protein synthesis inhibition, disruption of calcium homeostasis, and activation of pro-apoptotic receptor enzymes.[122,222] The spleen is a key location of immune system and lymphoid maturation. Thus, particle accumulation in the spleen may affect the

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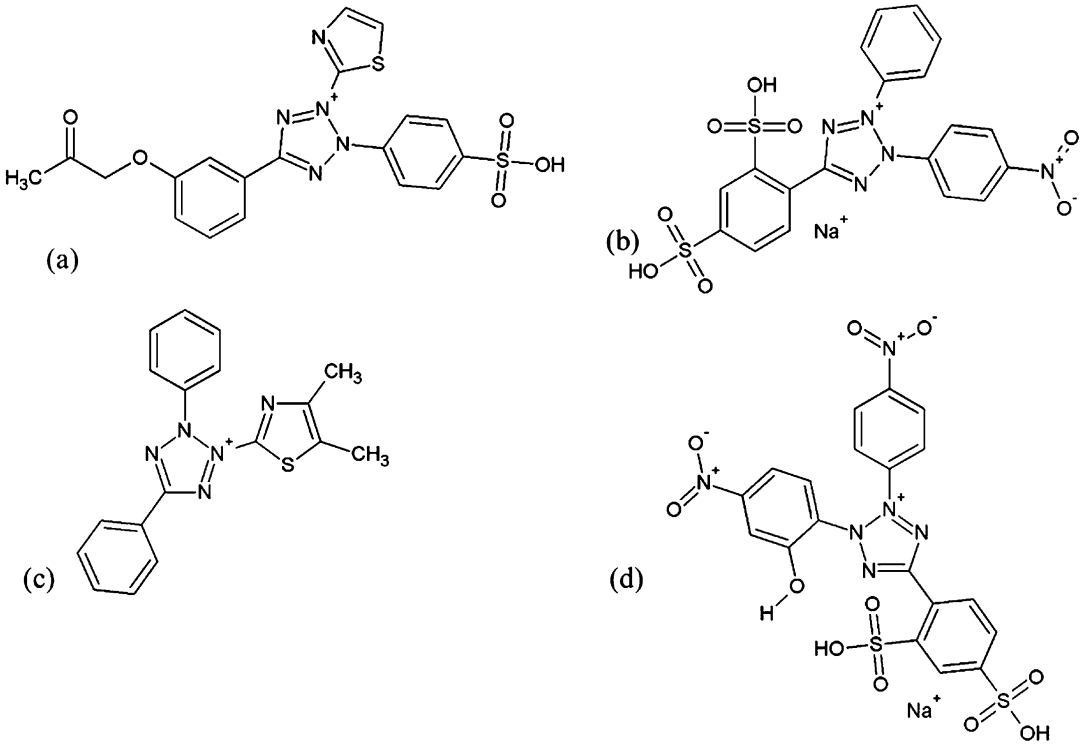

Figure 22. Tetrazolium salts: (a) MTS, (b) WST-1, (c) MTT, and (d) WST-8.

immune responses and immunopathology. In vitro analysis of spleen cells can help in understanding the toxic influence of the NMs. Studies illustrated the use of silica NPs for in vivo tumor imaging and studied the biocompatibility of the NPs by evaluating the toxic influence against spleen cells.[243]

■ ASSAYS FOR TOXICITY

Cytotoxicity Assay. *Basic Protocol for Cell Viability Quantification Using Different Colorimetric Assays.* The metabolic activity of the cells (viable or nonviable) leads to the release of several enzymes. Different dyes form complexes with enzymes released from the cells or with the DNA. These complexes generate different colors, and the intensity of the color helps in the further quantification of the number of viable or nonviable cells.[244−246]

In general, a given amount of cell suspension is mixed with a small amount of dye. Counting of cells is performed from a drop of this mixture generally using a hemocytometer and binocular microscope.[247−249]

The percentage of viable cells is calculated as follows:

$$\text{viable cells (\%)} = \frac{\text{total no. of viable cells per mL of aliquot}}{\text{total no. of cells per mL of aliquot}} \times 100 \quad (1)$$

Microculture Tetrazolium Assay. Microculture tetrazolium assay is a colorimetric assay meant for a qualitative cytotoxicity assessment. There are several types of tetrazolium salts such as 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2*H*-tetrazolium bromide (MTT), 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2*H*-tetrazolium, monosodium salt (WST-1), 3-(4,5-dimethyl-2-thiazolyl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium (MTS), 2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2*H*-tetrazolium, monosodium salt (WST-8) (Figure 22).[250,251] It is a nonradioactive assessment method which utilizes the metabolic activity of mitochondria as the parameter to evaluate the viability of the cell. A well-functioning cell has a mitochondrion, which is responsible for several metabolic activities inside the cell and releases numerous enzymes in this due course. In such a functional activity, there is a release of NADPH oxidoreductase enzyme.[252] This enzyme flows outside the cell membrane and interacts with the tetrazolium assay and converts into purple colored water insoluble compound formazan. Further, the compound is solubilized using dimethyl sulfoxide (DMSO).[253] The intensity of the color deepens with the number of viable or living cells; hence, varying the concentration can help us to get a quantitative estimation of the number of viable cells using a visible spectrophotometer.[91,254] However, this method is limited in quantifying accurately the exact numbers of viable cells. These assays have numerous pitfalls such as remaining insensitive to several human cell lines, and moreover, the risk of exposure of the lab technician to significant quantities of dimethyl sulfoxide cannot be ignored.[255−257] Hence, several modifications were implemented, and few tetrazolium derivatives such as XTT, WST-1, WST-8, etc. were synthesized, and they interacted with the cells to form water-soluble formazan.[258,259] Likewise, there exist a few reports of these colorimetric assays interacting with the nanoparticles and thus exhibiting exaggerated cell viability results (false reading) even at high doses of toxic exposure.[93,95,260−262]

Flow Cytometry. Flow cytometry is one of the most precise cell viability measurement techniques available. In a flow cytometer, the cell suspension is made to pass through a small slit which allows particles of 0.2−150 μm in size. The streamed liquid solution is interrogated using laser lights of definite wavelength. The laser light interacts with each cell passing through and scatters light. There are two types of scattered lights: forward scattered and side scattered lights. These two different types of scattered lights are detected by different detectors positioned at specific positions. The scattered lights are

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converted into voltage signals, where the higher the amount of scattered light, the higher is the intensity of the voltage signal gathered. The presence of any fluorescent molecule in the liquid suspension also fluoresces on interacting with the laser lights. This information gathered from the scattering of light and fluorescent light is utilized to understand the cellular kinetics, DNA/RNA content, enzymatic activity, etc.[263−266]

For cell viability measurements, a considerate amount of cell suspension is added with a small amount of propidium iodide (PI). PI is a nuclear stain dye; it is generally used to measure the apoptosis after the breakage of the cell membrane. It binds to the double-stranded DNA (of nonviable cells) and forms a fluorescent complex. This complex when excited at 488 nm gives emission at 617 nm. The amount of fluorescent light emitted defines the intensity of the peak obtained in the voltage pulse. The higher the number of fluorescent particles (nonviable cells) in the cell suspension, the higher will be the output intensity.[262] This method evaluates thousands of cells each second and provides a more consistent assessment. However, this method requires a complex and expensive instrument with a high maintenance cost per test and lengthy testing time.[267−269]

This is a new viability test that could be performed with the recently established microscopic cell counter and microchip (Adam, Nanoentek, Seoul, Republic of Korea). This instrument has implemented a direct cell counting technique for distinguishing viable and nonviable cells on a designed microchip with PI stain.[270]

Trypan Blue Exclusion Assay. Trypan Blue (TB) is a diazo dye used as a colorimetric assay to access cell viability (Figure 23). In this assay, cells are treated with trypan blue,

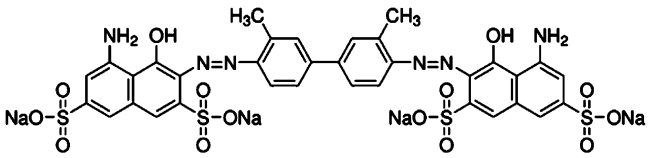

Figure 23. Chemical structure of trypan blue.

and it is further visually determined whether the cells uptake the dye or exclude it.[271] Unstained cells reflect the total number of viable cells and vice versa. A viable cell shall have a clear cytoplasm while the dead (nonviable) cell shall have blue cytoplasm. This assay is beneficial because it helps in the easy estimation of the actual number of viable cells, in contrast, to control, untreated cells, but the manual counting of the number of viable cells under the microscope makes the entire process tedious.[272]

Freitas et al. introduced the concept of using a regular TB assay and high precision flow cytometry for cell viability measurements. TB interacts with the cytoplasmic protein to form complexes. These complexes when subjected to green excitation light emit deep red fluorescence at 660 nm, which is detectable by flow cytometry.[273,274]

Clonogenic Assay or Colony Forming Efficiency. The clonogenic cell survival assay is another nonquantitative estimation to access the cell proliferation. In this assay, a given cell line is exposed to a specific dosage of toxic elements (nanomaterials, radiation, etc.) and allowed to proliferate for an extended period (1−3 weeks). The preferred cell lines are stained using gentian violet or nuclear stain and quantified per growth in number or size. It is assumed that each colony originates from a single plated cell, hence it is named as a clonogenic assay. A survival curve is plotted by placing the chances of survival (% of survival) across the increasing dosage of toxic element. Increasing the dosage of the toxicity gradually leads to lower the number (or size) of colonies. The clonogenic assay is also used for studying the effect of radiation, chemotherapeutic agents, and so on. The usual reports of interference of various colorimetric assays often showcase exaggerated viability measurements of the cells, and hence, this kind of qualitative estimation helps in verification of the viability assessment.[275−277]

Lactate Dehydrogenase Assay. Lactate dehydrogenase assay is another qualitative viability measurement technique. It is based on the amount of release of lactate dehydrogenase (LDH) enzyme. LDH is one of the cytoplasmic enzymes released after the breakage of plasma membrane due to multiple reasons such as necrosis and apoptosis.[278,279] Initially, the LDH releases nicotinamide adenine dinucleotide (NADH). The NADH released forms a colored formazan compound on interacting with basic tetrazolium salts. Later, the amount of formazan compound formed helps in the further quantification of the number of viable cells.[280] This assay appears to be convenient but has its own downsides. The release of the cytosolic enzyme cannot be a definite parameter to determine the cell death because at large there appear several instances when membrane damage or rupture might occur eventually. Thus, in such cases, cell viability measurements using such methods have higher chances of error. Hence, these assaying methods need to be verified using more quantitative viability techniques.[281]

Apoptosis-Detecting Assays. There are distinct types of cell death, as discussed in the earlier section (Various Kinds of Cell Death). Apoptosis, also known as programmed cell death, is generally characterized by condensation of the chromatin and the nucleus, blebbing of the cytoplasm, and DNA fragmentation. There are different apoptosis detecting assays, but in this review, we discuss only apostain, lamina-B and TUNEL technique.[282] The three techniques vary in principle yet give correlating results, as studied by Prochazkova et al.

Apostain Technique. Apostain technique detects the caspase-3 present in the cytoplasm of the cell which undergoes apoptosis. Apostain is a special mouse monoclonal anti-ssDNA antibody which detects cells with caspase-3 at a very early stage. Apoptotic cells stain brown, while the rest of the cells remain blue in color and are observed under a light microscope. The use of simple microscopy technique to assess viability makes this process exceptionally useful. Unlike other methods, apostain technique is much more sensitive, specific, and easy to apply and free of subjective interpretation. Moreover, it does not require a technician to recognize the apoptotic cells and does not depend on the DNA fragmentation, which is one of the late stages of the apoptotic process.[56,283]

Lamina-B Technique. Lamina-B technique is another early stage apoptosis assessment technique. The nuclear lamina is a mesh-like structure present between the inner nuclear membrane and heterochromatin. The nuclear lamins are responsible for different functions inside the nucleus such as DNA replication, chromatin organization, and so on. There are two types of lamins, lamin-A (acidic in nature) and lamin-B (neutral in nature). Initiation of apoptosis is marked by the release of a cascade of caspases. In such an event, caspase-6 is released, which is primarily responsible for lamin cleavage. Mutation of lamin A and B can trigger chromatin condensation and even DNA fragmentation. Hence, using immunohistochemistry, antigen markers are used to identify the lamin B.[284−286]

TUNEL Technique. DNA fragmentation is one of the final steps of apoptosis, and the TUNEL (terminal deoxynucleotidyl

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transferase dUTP nick end labeling) technique detects the DNA fragments. A fluorescent dye could be incorporated with the dUTP nucleotide present in the assay.[287] Thus, the cells with fragmented DNA get hitched by the assay molecules bound with the fluorescent markers, which can be further estimated using fluorescent microscopy or using immunohistochemical stains. This method appears to be very useful as it gives a more quantitative estimation of the number of viable cells. However, it suffers from few shortcomings, as it fails to distinguish between apoptosis and necrosis and only detects at the final stages of the cell damage.[288,289] Unlike conventional assays, there exist different other techniques to count or measure the number of viable cells.

Cell Proliferation Assays. Cell proliferation assays are used to access the cell viability after exposure to toxins. It even helps in early detection of several types of cancer. There are different methods to analyze the cell proliferation such as histochemical, immunohistochemical, and flow cytometric approaches. In the histochemical assaying technique, the DNA content of the cells subjected to viability assessment is measured directly by staining them with different kinds of fluorescent or radioactive markers such as [^{3}H] thymidine and bromodeoxyuridine. Measuring the DNA content is one of the most accurate and reliable techniques available. [^{3}H] thymidine, in this approach a radioactive thymidine analogue, is replaced in the DNA of a viable cell during the mitosis process.[290] The thymidine uptake in the cell is replicated and helps in the assessment of the viability of the cells. The radioactivity of the DNA obtained from the cells after cell division is measured using a scintillation beta-counter, which help in the quantification of the number of viable cells. This method of thymidine uptake can be readily used in immunohistochemistry and immunocytochemistry assays. However, there are few demerits of this assaying technique. Incorporation of radioactive thymidine might lead to DNA mutation and damage. Moreover, sometimes the dividing cells in mitosis do not replicate the thymidine added, and this replication process cannot be controlled in vitro. The addition of radioactive materials is quite expensive and requires additional facilities and training. Bromodeoxyuridine, 5-bromo-2′-deoxyuridine (BrdU), is a nonradioactive thymidine analogue incorporated into synthesizing DNA. This marker is utilized to measure the cell cycle kinetics. It is detected using anti-BrdU antibodies. This marker is more efficient than [^{3}H] thymidine, but it too suffers from a few disadvantages. The use of anti-BrdU antibodies for assessment leads to denaturation of DNA and destroys the DNA morphology. There exist a few other markers such as 5-iodo-2′-deoxyuridine (IdU) and 5-chloro-2′-deoxyuridine (CldU), which also serve as thymidine analogues and are incorporated into newly synthesized DNA. As with BrdU, they too suffer from the same set of shortcomings.[291−293]

Another advanced proliferation assaying technique is to analyze the proliferation proteins. Identifying proliferation proteins can help to distinguish cells undergoing proliferation from the nonproliferating ones. This assaying technique requires primary antibodies against the antigens expressed during proliferation.[294] These antigens are typically present at all the stages of cell division, thus making them excellent markers. Ki67, PCNA, and MCM-2 are the prominent antigens used for this purpose. These markers help in diagnosis and even prognosis of several types of cancer at a very early stage.[295−297]

Genotoxicity Assays. The ability of several toxic agents to alter the genetic information causes a mutation which leads to cancer. However, all the mutagens are genotoxic, but all genotoxicants are not mutagens. There are several methods available to access the genotoxicity of materials. In this assaying technique, the researchers are keen to identify any kind of DNA damage caused due to toxic insult. The DNA damage could be the rupture of any DNA strands, point mutation, or chromosomal aberrations.[298,299]

COMET Assay. The single cell gel electrophoresis assay (SCGE) or the comet assay is one of the widely used in vitro assays for detecting DNA single strand breaks, alkali labile sites, and cross-linking with the single cell approach typical of cytogenetic assays. It is one of the sensitive, reliable, and inexpensive methods for DNA damage analysis. This assay is based on the principle of DNA fragment separation using gel electrophoresis. Negatively charged DNA fragments are drawn through an agarose gel under the influence of an electric field. Cells encapsulated in an agarose solution are further lysed using detergents and salts. The lysis of the cell with nonionic detergent and high-molarity sodium chloride leads to complete digestion of cytoplasm, membrane, mitochondria etc. and only left with nucleoid-containing supercoiled negatively charged DNA, RNA, and protein. Further electrophoresis at high pH results in a structure like a comet; staining those using fluorescent markers helps them to be visualized using fluorescent microscopy. The head of the comet is composed of the intact DNA and the tail consists the damaged DNA fragments. The relative intensity of the tail of the comet to its head accounts the damage caused in the DNA. The possible reason for formation of the comet-shaped structure while migrating the DNA in an electric field can be attributed to the attraction of broken (loosened from the supercoiling) DNA fragments to the anode.[300−303]

AMES Test. It is yet another type of in vitro assay that is utilized to access the genotoxicity of materials. Because the chemical composition of DNA is the same in all animals, therefore any organism or animal can be used to evaluate the toxicity ability of any source. Ames and colleagues utilized this idea to form a reliable and quick procedure for genotoxicity assessment. In this assay, mutant strains of the bacteria *Salmonella typhimurium* (*S. typhimurium*) is used to evaluate the toxicity ability of any source. This bacterium already has a mutation in the gene encoding for histidine enzyme where it is deficient of histidine production, but it requires histidine for growth. Hence, when subjected to potential toxic insult, if the bacteria undergo mutation again (reverse mutation) of the gene encoding for histidine enzyme to enable its production. This process of reverse mutation can aid in the identification of potential mutagen or carcinogen. Although this method serves its own demerits, the use of bacterium strain (a prokaryote cell) cannot be precisely used as a model for human cell metabolism. Hence, in several instances, rat liver cells are used to mimic human cell structures.[304,305]

Measurement of Oxidized Guanine Bases. The oxidative DNA damage is considered one of the main causes of cancer. Reactive oxygen species such as hydroxyl radical, hydrogen peroxide, singlet oxygen produces superoxide radicals. These superoxide radicals remain inactive toward DNA directly but serve indirectly to activate oxidation of the guanine bases present in the DNA strands and even cause rupture to these strands. Hence assaying the oxidized base pairs can help to assess the toxicity potential of the subjected insult. The most common are the measurement of 8-hydroxydeoxyguanosine (8-OHdG) and 7,8-dihydro-oxodeoxyguanine (oxo-dG) by HPLC with electrochemical detection (HPLC-ECD).

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These bases are the products after undergoing oxidative damage. However, it might lead to a problem if the bases undergo incomplete enzymic hydrolysis and then shall interfere with the action of nucleases and deglycosylation of 8-OHdG during sample handling.[306,307]

Chromosomal Aberrations Assays. Analyzing the chromatic aberration is another parameter of genotoxicity measurement. Evaluation of the chromosomal integrity is done using different types of assays. Unlike genomic assays, the chromosome assays help in visualization of the integrity of the chromosomes. There are two types of structural aberration; chromosomes or chromatid. There are different chromosomal aberration assaying techniques as discussed below.[308,309]

Micronucleus Assay for Chromosomal Aberrations. In this assay, micronuclei are searched in the cell. Micronuclei are small chromatin-containing bodies comprising partial or whole chromosomes left out from the nucleus after being subjected to toxic insult. This assaying technique can be used for preliminary assessment technique for carcinogens.[310]

Giemsa or Giemsa/Trypsin Staining. This assaying technique helps in visualizing the structural integrity of the chromosomes. Staining the chromosomes with Giemsa turns them into purple. Treating the chromosomes with proteolytic enzyme trypsin and then adding Giemsa provides more enhanced visualization by forming typical G-banding.[311]

RECENT ADVANCES IN TOXICOLOGICAL ASSESSMENT OF NANOMATERIALS

Industrial manufacturing procedures often convert engineered nanomaterials into extremely toxic elements. However, the risk of exposure to such components depends on the probability and the extent of their exposure to living cells.[312] Thus, toxicity studies (in vitro and in vivo) helps to ascertain the toxicity potential. The toxic potential of several nanomaterials depends upon set of intricate factors as discussed in the previous section; hence, screening methods certainly turn out to be the decisive feature. In consequence, the present section is a discussion of a summary of the recent advances in toxicological assessment in past few years and provides a brief description of those works.

Metal Nanoparticles. The wide use of metal nanoparticles has implored its need for toxicity studies to be performed.[313,314] Studer et al. studied the influence of solubility in the toxicity impact on living cell type (CHO and HeLa). The authors compared the toxicity of copper NPs having distinctly different chemical and physical properties. The cytotoxicity of copper coated with carbon and copper oxide is evaluated. The carbon coated CuO NPs showed controlled toxicity because of the surface properties, while the Cu NPs showed the Trojan horse type mechanism and induced significant toxicity. This study again proved the relevance of physicochemical property.[315] Ortega et al. evaluated the significance of Trojan horse type mechanism of toxicity induction by less soluble nanomaterials. The study found that the less soluble copper oxide NPs were up taken inside the cell by clathrin-dependent pathway. However, they get partially soluble at low pH environment of lysosomes to release Cu ions, which further commit to the cytotoxicity.[316] In a different study, the authors evaluated the influence of media in the cellular uptake and cytotoxicity of Au nanoparticles. The NPs on entry to the cellular medium forms NP−protein complex, thus changing the media led to formation of different type of complex. Dulbecco's modified Eagle's medium (DMEM) and Roswell Park Memorial Institute medium (RPMI) were used. The RPMI formed a complex with the NP which showed greater intake and higher levels of toxicity compared to DMEM.[317] Cao et al. inspected the toxicity of two different consumer creams containing Au NPs. It was observed that the NPs did not induce any toxicity to the exposed cell lines even after 24 h.[318] Siddiqi et al. studied the toxicity profile of the Au NPs in rat brain. Au NPs triggered generation of oxidative stress and a reduction of antioxidant enzyme, such as the glutathione peroxidase activity in rat brain. There was also increase in 8-hydroxydeoxyguanosine, caspase-3 and heat shock protein70, which increased the chances of DNA damage and cell death.[319] Park et al. studied the cytotoxicity of silver nanoparticles on mouse peritoneal macrophage cell lines. (RAW264.7) with dosage up to 1.6 ppm and exposed for 96 h. Ag nanoparticles were internalized via phagocytosis and induced toxicity by Trojan horse type mechanism. The cellular viability decreased in dose and time dependent manner. Moreover, the toxicity of the Ag NPs was attributed to the enhanced ROS levels and inflammation.[320] In another report, the authors studied the difference in the toxicity effects of Ag nanoparticles and Ag ions. The Ag nanoparticles induced considerable levels of toxicity inside the exposed cells. The introduced nanoparticles caused alteration in histone methylation, which further reduced the hemoglobin levels, while the Ag ions did not contribute anything to the histone alteration.[321] Silva et al. compared the in vivo pulmonary effects of post instillation by short and long nanowires. It was observed that the short and the long nanowires induced pulmonary toxicity.[322] In vivo studies of Au NPs distribution was studied using different analytical techniques such as inductively coupled plasma-mass spectrometry (ICP-MS), TEM, EDX (energy-dispersive X-ray spectroscopy), and X-ray absorption spectroscopy (XAS). The results found that the Au NPs were localized in the liver and spleen tissue of Sprague−Dawley rats.[323] In a different approach, Hainfeld et al. studied the use of Au NPs as X-ray contrast agent in Balb/C mice. Deliberate addition of 100 μg/mL of gold nanoparticles provides good contrast-to-noise image. The gold nanoparticles are useful X-ray contrast agents that offer novel physical and pharmacokinetic advantages over current agents.[324] Apart from toxic influence toward mammalian cells, the nanoparticles also have illustrated bactericidal behavior against wide range of microorganisms. A recent review by our group highlights the major studies exhibiting the same, which have been discussed in detail.[325] The use of this bactericidal nature of the materials has found productive applications in the health care sector and potential self-cleaning antimicrobial applications. The light activation of several semiconductor nanomaterials produces active ROS which significantly affect the viability of the exposed cells.[326] In this aspect, Ritmi et al. recently examined the cytotoxic nature of Cu-TiO_2 coatings. The synergistic effect of Cu and TiO_2 contributed toward the bactericidal behavior.[327] Composites of semiconductor materials with metal nanoparticles decreases the overall bandgap of the photocatalytic composite and the recombination rate is also reduced.[328] Magnetic composites have attracted attention of several industries because of their easy retrieval after the application and their attitude toward reusability. As observed in case of mammalian cells, the bacterial cell walls are tampered by the aggregates of the nanomaterials. Moreover, the leaching of the metal ions is also a critical contributor to the bactericidal behavior.[329,330] Raut et al. recently reported the formation of chitosan-TiO_2:Cu nanocomposite for biomedical applications. The as-prepared composite displayed a 200% improved inactivation of bacterial strains of *E. coli* and *S. aureus*, compared to

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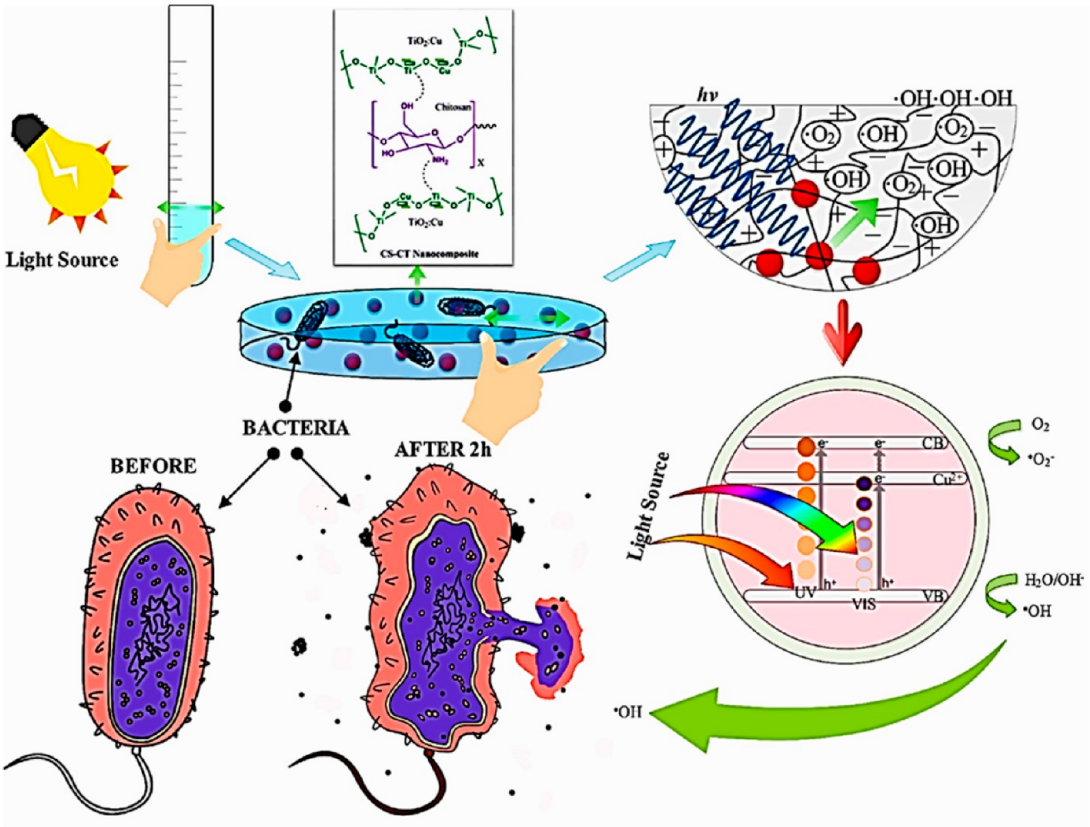

Figure 24. Illustration of the plausible mechanism of antimicrobial activity in the presence of nanocomposite. Reproduced with permission from ref 331. Copyright 2016 Elsevier.[331]

chitosan only inactivation samples. The possible mechanism of the disinfection process is illustrated in Figure 24.[331]

Metal Oxides. Metal oxides are an important category of industrial materials used frequently as semiconductor applications, as catalysts for redox reactions etc. Toxicity induced by metal oxides have been studied widely, and the possible explanations behind the enhanced toxicity levels were already discussed in Nonoxidant Routes to Cellular Injury.[332] However, in this section the review focuses on some recent studies on metal oxide-based toxicity.

Lu et al. studied the toxicity influence of varied range of metal oxides. The in vitro study used LDH assay on exposed human epithelial cell lines (A549) and observed that the ROS production increases with the increase in the surface area, which necessarily increased the toxicity imposed.[333] Zhang et al. studied the influence of band gap and band edge potential of metal oxides to predict the nature of the oxidative stress and pulmonary inflammation. The authors found that the metal oxide particles completely soluble in the biological environment and has a band gap value comparable to the cellular redox potential (−4.12 to −4.84 eV) exhibited high levels of toxicity.[334] CeO_2 like any other rare earth metal have trivalent oxidation state but it has also +4 oxidation state and as any other metal oxide is expected, it as well imposes acute levels of toxicity.[335] However, Chen et al. studied the effect of its structure for its antioxidant property.[336] Owing to its flip-flop from +3 to +4 oxidation states, because of several surface chemical reactions lead to the formation of defects by sacrificing any oxygen or electron. These defects are very spontaneous and are altered by minor changes, alike any stress or presence of any ions in the environment and behave as traps for various ROS. Thus, because of its physicochemical behavior, ceria prevents the cell from the oxidative damage. In the same study, they defined that the defects increased with the increase in surface area and thus nano ceria has a number of defects. Their behavior as antioxidants was further studied by Ji et al.[173] In a recent effort, a group studied the in vitro toxicity effects of Zn doped Ceria in cell lines of Neuro2A. The composite exhibited dose dependent toxicity up to a concentration of 31.25 μg/mL.[337] Auffan et al. evaluated the influence of the redox state of iron-based nanoparticles and their cytotoxicity toward strains of *Escherichia coli*. Maghemite (γ-Iron), Fe_3O_4, and zerovalent Iron were the materials for assessment. The stable γ-Iron did not impose any credible toxicity but the other two ions of Iron (Fe^{2+} and Fe^0) exhibited significant levels of toxicity due to oxidative stress. Iron undergo fenton reaction while reacting with the oxygen in the cellular environment and produces ROS.[338] Sisler et al. studied the toxicological assessment of CoO and La_2O_3 on strains of human small airway epithelial cells (SAEC) of dosage up to 50 μg/mL and exposed for 24 h. This study showed that Lanthanum oxide showed dose dependent toxicity and the toxicity was only observed after 24 h. It is comparatively less toxic than other metal oxides.[339] Two different types of mesoporous silica NPs were loaded with curcumin and evaluated for target drug delivery system (Figure 25). Bollu et al. studied the cytotoxic properties of curcumin-loaded silica-based mesoporous materials against cancer cells. The NP system did not induce any credible toxicity when exposed to CHO cells but at the same time the system induced extreme levels of toxicity when subjected to different cancerous cell lines.[340]

Thai et al. investigated the toxic influence of six different titania nanomaterials on human liver HepG2 cells. The study

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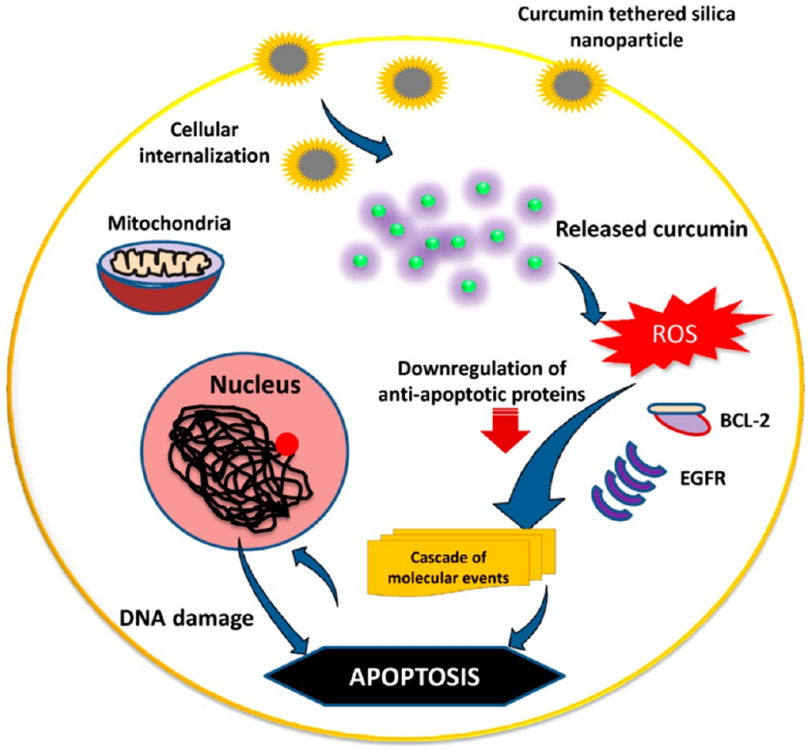

Figure 25. A mechanistic route for anticancer application using curcumin loaded mesoporous silica materials in cancer cells. The intake of curcumin loaded silica-based materials results in the discharge of curcumin in the cytosol. Free curcumin then interacts with some specific molecular targets causing the production of reactive oxygen species which reduces the regulation of antiapoptotic proteins BCL-2 and EGFR triggering the apoptotic pathway. Reproduced with permission from ref 340. Copyright 2012 Elsevier.[340]

aims to derive the possible signaling pathway by different titania on interacting with the human HepG2 cells. The protein ubiquitination, hepatic fibrosis, and cancer-related signaling pathways were tampered in this process. The influence of the NPs to modify the gene expression is determined by the hydrodynamic property rather than the dry particle size.[341] Vergaro et al. evaluated the cytotoxicity of different titania nanoparticles on human bronchial epithelial cells (BEAS-2B). The cytotoxicity of anatase titania was compared with P25 particles (Standard TiO_2 nanoparticles with mixed anatase and rutile phase). The P25 particles were found to be cytotoxic even at a very low dosage. The modified titania samples however showed low levels of toxicity.[342] Ren et al. studied the drug (doxorubicin) delivery ability of black TiO_2-based core−shell nanocomposites. Mesoporous silica coated with black TiO_2 help in controlled NIR (Near infrared) triggered release of drug. The viability measurements carried out against human breast cancer cell lines showed appreciable biocompatibility.[343] Tassinari et al. studied the oral, short-term exposure to titanium dioxide nanoparticles on reproductive, endocrine systems and spleen in sprague-dawley rat. Oral exposure to anatase TiO_2 for a short duration exhibited observable reproductive effects in the rat cells of thyroid and adrenal medulla etc. But, the titania levels remained low inside the tissues.[344] Dubey et al. studied the oxidative stress and nanotoxicity induced by TiO_2 and ZnO on WAG cell line. The NPs exhibited acute levels of toxicity and illustrated a dose dependent increase in the toxicity. The authors measured the lipid peroxidation by 'Thiobarbituric Acid Reactive Substances' (TBARS) assay. The Malondialdehyde formed due to the lipid peroxidation forms adduct with TBARS which is calorimetrically active compound. Therefore, quantitative estimation of absorbance helps in the estimation of the toxicity imposed.[345,346] Similarly, in another study, Leung et al. studied the toxic influence of titania and ZnO against the bacterial strains of *E. coli*. The authors evaluated the lipid peroxidation by using TBARS assay. The titania NPs exhibits higher levels of toxicity compared to ZnO at the same concentration. TiO_2 NPs showed high attachment and eventually exhibited reduced rate of cell proliferation.[347] Song et al. investigated the cytotoxicity of ZnO nanoparticles using strains of Ana-1 cell with CCK-8 assay and fluorescence assay. The study aims to find the importance of dissolved zinc ions in the cytotoxicity of the cells. It was observed that the ZnO NPs showed dose dependent toxicity and the shape of the particles played less significance. However, the ROS species were produced predominantly because of the dissolution of Zn ions and were less dependent on the presence of the ZnO NPs.[348] In a recent effort, Delaval et al. evaluated the toxicity of ZnO nanoparticles using the human bronchial epithelial cell line NCI-H292. The oxidative potential of the NPs was assessed using cytochrome c assay. The ZnO NPs showed increase in toxicity by dose dependent manner. The study aims to consider cytochrome c assay as a credible measurement technique to evaluate the oxidative potential of different materials.[349] Similarly, another group of researchers explored the toxicity profile of ZnO quantum dot. It was observed that the ZnO QDs show greater cytotoxicity against MCF-7 and metastatic MDA-MB-231 human breast cancer cells. The images obtained from confocal microscopy and TUNNEL assay revealed that ZnO QDs induced nuclear fragmentation and apoptosis in the cell lines.[350] Bacchetta et al. studied the influence of soluble zinc in ZnO nanoparticle toward the cytotoxicity in *Daphnia magna*. The Zinc ions leached from the ZnO NPs are ingested inside the cytosol and which further tampers the mitochondrial

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permeability. The solubilized zinc ions increase the ROS generation and further disrupts the cellular metabolic activities.[351] In a recent effort, Jeyabharathi et al. found that the ZnO NPs imposed dose dependent toxicity on the embryos of zebrafish. The authors attributed the toxicity to the leaching of Zn ions.[352] In another study, the authors synthesized two different morphologies of ZnO nanowires and evaluated the biocompatibility behavior of the same by trypan blue assay and flow cytometer. HEK293 cells were grown over fan shaped and vertical shaped nanowires. The fan shaped showed increased cytotoxicity compared to vertical shaped nanowires. This result again emphasized the importance of morphology in the toxicity profile of nanomaterials.[353] As discussed in the previous section, the toxicity profile of metal and metal oxide NMs essentially contribute toward productive applications. In a recent study, Nesic et al. reported a composite of TiO_2 with polyester. The as-prepared composite illustrated complete bacterial inactivation within 2 h of exposure in the absence of light. The plausible mechanism based on the TEM reports define the rupture of the bacterial cell wall by the titania aggregates.[354] Leyland et al. studied the bactericidal effect of TiO_2 coatings doped with Cu and F against the disinfection of strains of *S. aureus* (ATCC 6538). The coatings fabricated illustrated more than 4 log reduction under visible light irradiation (Figure 26).[355]

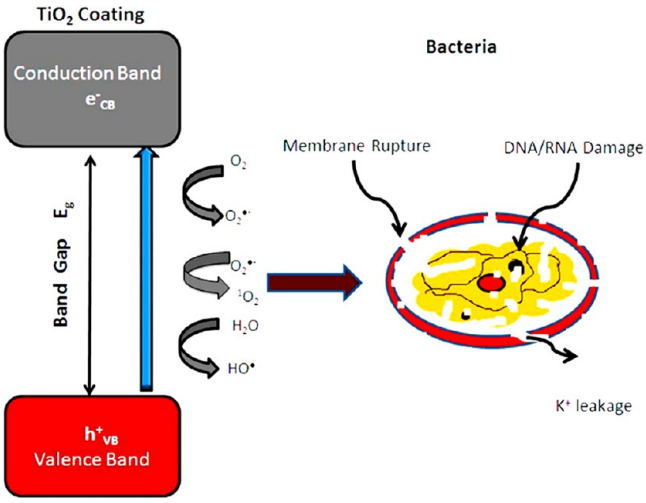

Figure 26. Schematic representation of the photocatalytic antibacterial action. Reproduced with permission from ref 355. Copyright 2016 Nigel S. Leyland, Joanna Podporska-Carroll, John Browne, Steven J. Hinder, Brid Quilty, and Suresh C. Pillai.[355]

Carbon Nanotubes (CNT). Carbon nanotubes are used exhaustively for different applications because of their unique electronic and physical properties. Based on the number of layers, CNTs are classified into two types, Single wall carbon nanotube (SWCNT) and Multi wall carbon nanotube (MWCNT).[356] The influx of potential applications using CNTs have persuaded researchers to look upon the potential toxicity of these materials. There exist several reasons for toxicity as assessed in different studies. Few reports suggest the presence of metal catalyst particles (impurities) on CNT as the reason behind the acute levels of toxicity. However, there remains a definite lack of research to ascertain the prime reason. However, it is impossible to synthesize CNTs devoid of metal particles entirely.[357]

Magrez et al. studied the significance of the aspect ratio of CNTs in their cytotoxicity potential.[176] The study found the cytotoxicity of the materials in the order of carbon black > carbon nano flakes > carbon nanotubes. The filaments were observed to be less toxic than the particles. The morphological alterations of the cells after a few days of exposure with all the materials, remained the same but the difference in the viability was attributed to the variance in the interaction of the NMs with the exposed cells. Moreover, the difference in the interaction of the cells and the NMs was presumably ascribed to the presence of dangling bonds. These bonds are high reactive sites which are found in high densities on carbon black and found least in CNTs. Conversely, Isobe et al. researched the estimated cytotoxic potential of the CNTs and found them to have only small levels of toxicity.[358] There are several studies indicating conflicting results on the cytotoxicity potential of CNTs. In fact, Knirsch et al. have tried to critically examine the cytotoxicity standards for assessing nanomaterials.[359] It has been clearly assessed in this study that CNTs show low levels of toxicity and assaying nanomaterials using MTT may not be reliable, as there exist several instances of assay interference. Additionally, Kang et al. studied the antimicrobial property of SWCNTs and attributed the direct interaction of the SWCNTs with the cell membrane as the prime reason for low cell viability.[356] Conversely, Vecitis et al. later differed the assessment by proving the electronic structure of the CNTs to be the reason behind the bacterial cytotoxicity.[357] There exist several mechanisms illustrating the potential routes to induce toxicity by these NMs. Liu et al. in their study summarized the toxicity studies of CNT and explained several disparities in the result observed in the toxicity assessment.[360] Oxidative stress is one of the prime reasons for toxicity in the cells and the ROS produced has the potential to interfere with the cellular metabolism. The transition metal species present in the CNT as impurities also avails the potential to induce considerable levels of toxicity in the exposed cells. A summarized view of the potential toxicity mechanism is provided in the schematic below Figure 27.

Guo et al. demonstrated the use of PEG functionalized SWCNT for targeted drug (dopamine) delivery. The SWCNT nanomaterials show appreciable cellular membrane penetration ability, with high drug loading capacity and pH responsive unloading ability. The in vitro results helped in optimizing the dosage of the functionalized drug carrier. The PC12 cells were treated with different dosage of nanomaterials and further the toxicity assessments were performed using MTT, LDH, ROS assay. The results found 6.25 $\mu g\ \mu L^{-1}$ as the optimum dosage for the release of dopamine. *In vivo* estimation also validated the same results and found the optimum dosage to be 3.25 mg kg^{-1}.[361] Li et al. performed an in vivo study to understand the effects of perfluorooctanesulfonate with single wall carbon nanotubes (SWCNT) in zebrafish. The samples were exposed for 24, 48, 72, and 96 h separately. The results showed the bioaccumulation of perfluorooctanesulfonate in liver, intestines, gills and brain of fish with an increase dosage of SWCNT.[362] Reports of Titania/carbon nanotube heterojunction by Akhavan et al. and their application illustrating the cytotoxic nature against bacterial cells (*E.* coli) under visible light irradiation. The Ti−C and Ti−O−C carbonaceous bonds developed efficiently aided to the effective visible light absorption and ultimately added to the improved bacterial inactivation.[363] Recently, Koli et al. examined the bacterial disinfection efficacy of TiO_2/MWCNT (Multiwalled carbon nanotube) composite against the strains of *Escherichia coli* and *Staphylococcus aureus* under visible light irradiation. The composite displayed enhanced disinfection paralleled to the TiO_2 samples due to the effective charge separation imposed by MWCNT.[364]

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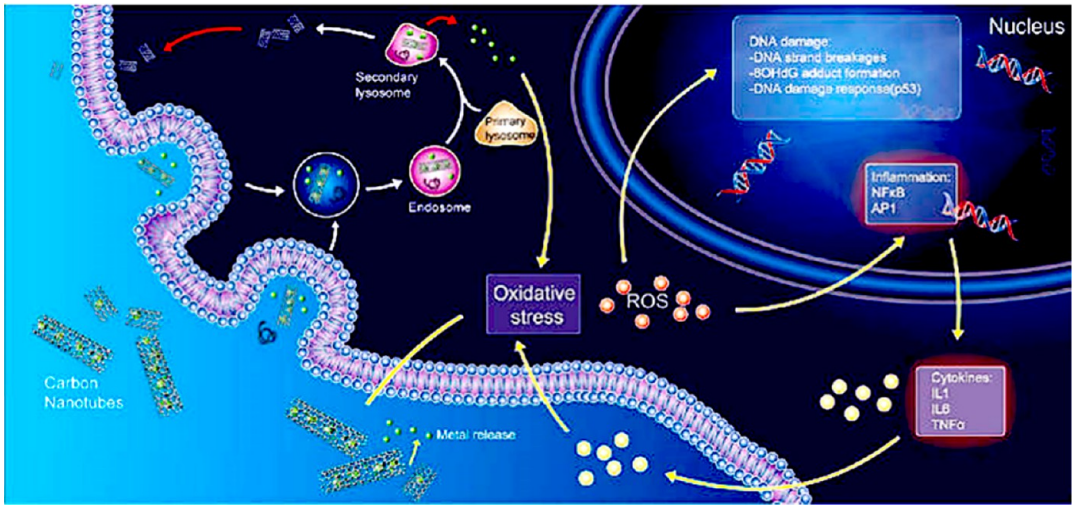

Figure 27. A summarized view of the potential toxicity mechanism. Reproduced with permission from ref 360. Copyright 2012 American Chemical Society.[360]

Graphene and Graphene Oxide (GO). This 2D class of nanomaterials have shown promising results against various applications.[365,366] Hu et al. studied the antibacterial behavior of a monolayer of graphene. The monolayer showed a very low level of toxicity. The authors also managed to fabricate free-standing paper coated with graphene oxide and reduced graphene oxide using simple vacuum filtration technique. This eventually laid the foundation to more optimistic plans of utilizing graphene for various clinical and environmental applications.[367] Teo et al. evaluated the toxicity induced by halogenated graphene. The halogenated graphene induced significant amount of toxicity and the toxicity amount increased with the increase of halogens content in the graphene sheets.[368] In another study, the same authors contradicted the above inference about the increased cytotoxicity to the increased content of halogen atoms. In this case, the fluorographene did not exhibit increased toxic levels; on the contrary the increase in fluorine content did not increase the toxicity but decreased it by 2 to 3 fold. The authors ascribed the difference in the behavior to the presence of a greater number of mono substituted carbon atoms (due to F atoms) in the graphene sheets.[369] Bengtson et al. found that graphene and graphene oxide do not induce any cytotoxicity nor genotoxicity in murine lung epithelial cells (FE1). The study found no change in the cell proliferation levels when exposed for 24 h with a maximum 200 μg/mL of GO and graphene (with lateral size less than 0.5 μm). The cells were analyzed using DCFH-DA (oxidation assay) and COMET assay.[370] Hu et al. studied the influence of cell growth media and the eventual protein coating around the cells, to understand their ultimate effects on imposing toxicity. The GO induced significant amount of toxicity upon interaction with the cells but the viability did not change after a few hours, and it apparently remained constant. The formation of an NP−protein complex was attributed to be the prime reason. On coating GO with fetal bovine serum (FBS), the cell proliferation was not affected at all. This verified the influence of the complex formation of GO with the components of the medium.[371] Torres et al. studied the cytotoxicity and internalization of two different types of GO nanoparticles. Low-reduced GO (LRGO) particles were synthesized and their toxicity was compared along with GO. The LRGO particles showed 5 times more toxicity than the GO. The surface chemistry and the size of the particle were the reason behind the increased toxicity.[372] Like GO, the composites of graphene oxide had a mixed results on their impact of toxicity. Luna et al. reported GO/Ag composite, which exhibited increased cytotoxicity compared to GO after exposing the J774 macrophages cells with a dosage up to 100 μg/mL for 24 h and further evaluating with ICP-OES and dynamic light scattering (DLS). The high oxidative stress induced by the composite is attributed to be the reason behind the increased toxicity.[373] Similarly, another report by Yan et al. demonstrated a comparable toxicity of the GO/Fe_2O_3 composite and GO itself.[374] On the other hand, reports of GO as a biocompatible material have been also reported. Isis et al. synthesized a polymer−graphene oxide composite and evaluated the cytotoxicity of the as-prepared composites on NIH 3T3 fibroblast cells lines (dosage up to 1000 μg/mL and exposed for 24 h). The composite prepared had only 3 wt % of GO but showed excellent antimicrobial behavior and significantly low toxicity.[375] Likewise, zinc(II)-loaded zeolite/graphene oxide nanocomposite was reported as a new drug carrier. The composite prepared was evaluated for their cytotoxicity against the A549 cell lines (dosage up to 0.1 mg/mL and exposed for 24 h). The composite shows extremely low toxicity and was found to be biocompatible.[376] Another report by Bahamonde et al. illustrated the biocompatibility of reduced graphene oxide on functionalization with polysulfone brushes which necessarily enhanced its antibacterial properties and reduced human cytotoxicity.[377] Yue et al. reported a bifunctional GO composite with PEG and polyethylenimine (PEI) for gene editing in human cells using Cas9/sgRNA (Figure 28). The Cas9/sgRNA interacted by π interaction and further the nanocarrier was encapsulated inside the cell using endocytosis. The authors evaluated the cytotoxicity of the nanocarrier using MTT assay. AGS-EGFP cells delivered with Cas9 of concentration as high as 120 μg/L were exposed for 48 h. The cellular viability was observed to be approximately 95%, and this proved the biocompatibility of this composite for cell imaging and drug delivery component.[378]

In vivo studies of GO were effective to understand the localization and distribution of the nanomaterials as reported.[379] Yang et al. studied the efficiency of graphene uptake and as an efficient photothermal therapy agent. The nanographene sheets (NGS) coated with PEG showed improved biocompatible

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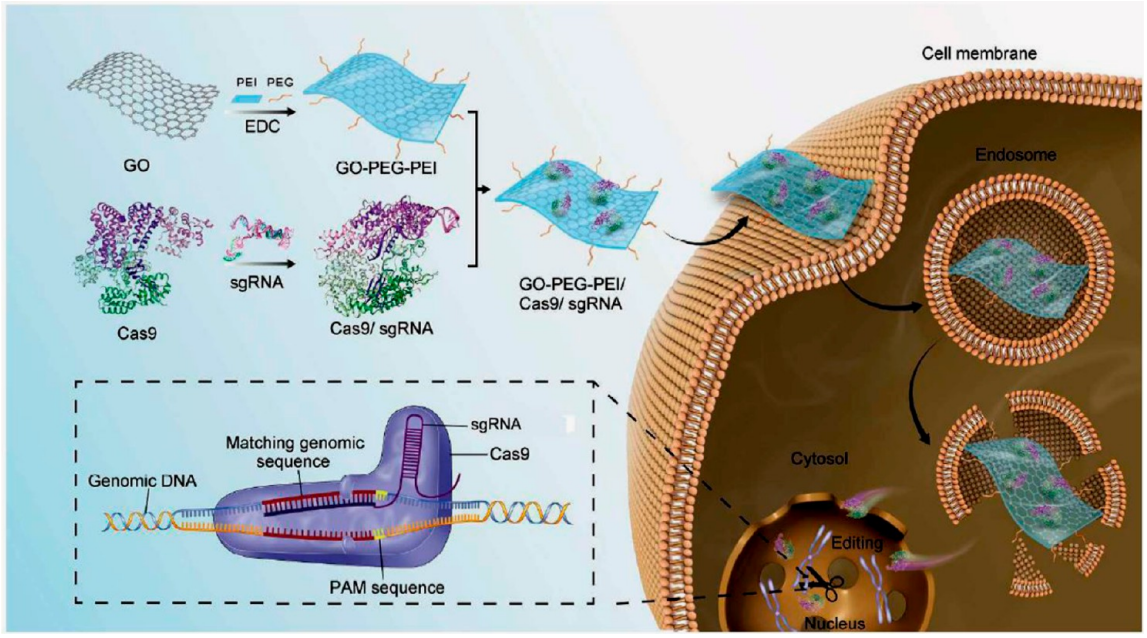

Figure 28. Schematic diagram of the GO−PEG−PEI-based Cas9/sgRNA delivery system. The GO−PEG−PEI was loaded with the Cas9/sgRNA complex via physisorption and π-stacking interaction to form GO−PEG−PEI/Cas9/sgRNA complex. Subsequently, the complex was delivered into cells, and the processes are as follows: binding to the cell membrane, endocytosis, endosome escape, transport into the nucleus, search for the target DNA locus in the chromosome, and introduction of double-strand breaks for gene editing. Reproduced with permission from ref 378. Copyright 2018 The Royal Society of Chemistry.[378]

properties than CNTs. It showed low reticuloendothelial system (RES) accumulation and notably improved tumor passive targeting effect (Figure 29). It appeared to be an exceptional in vivo tumor near-infrared (NIR) photothermal therapy agent without displaying visible toxicity to the treated mice.[380]

The same group also studied the pharmacokinetics and the quantitative in vivo biodistribution of the PEG coated graphene nanosheets. The functionalized graphene mainly accumulated in the RES, including the liver and spleen; however, it was gradually cleared by both renal and fecal excretion.[381] Another report also investigated the biodistribution of the graphene nanoplatelets. The graphene nanoplatelets showed an absence of any acute or chronic toxicity, and moreover, they did not show any levels of genotoxicity.[382] In such a different study, the authors evaluated the pharmacokinetics and the quantitative in vivo biodistribution of the PEG coated GO. The results revealed that the PEG coated GO after oral administration did not get adsorbed in any organs and was excreted quickly; on the other hand, when it was injected intraperitoneally, the functionalized GO was engulfed by the phagocytes and got accumulated in the RES system. The intake and the accumulation of these materials was dependent on its size and its shape.[383] Cho et al. studied the in vivo comparison of the immunotoxicity of single- and multilayered graphene oxides with or without pluronic F-127. A toxicity evaluation in the acute and chronic phases was performed in mice via intravenous injection of graphene oxide. The single and multiple layered GO induced substantial inflammation at acute phase. Additionally, various degrees of renal fibrosis and inflammation was observed in the chronic phase. Multiple layered GO induced more inflammation than the single layered GO.[384]

Novel Materials. Layered materials such as bismuth oxyhalide had recently acquired attention due to its impressive semiconducting behavior. Xu et al. in a latest effort, studied the toxicity influence of bismuth oxychloride using in vitro cytotoxic assay (MTT) on human breast cancer cell line (MCF-7). The cell viability was observed to be around 80% when exposed for 24 h; however, the cell viability was reduced to 10% when the exposed cells were simultaneously irradiated with UV light.[385] Teo et al. studied the cytotoxicity of exfoliated MoS_2, WS_2, and WSe_2 using human lung carcinoma cells (A549). The study revealed that the level of toxicity follows the order $WSe_2 > MoS_2 > WS_2$. WSe_2 is considered the most toxic among the given TMDs. The possible hypothesis to this difference in the cytotoxicity levels is attributed to the presence of chalcogenides in the outer layers of the TMDs. They are the elements interacting with the cells directly. Earlier studies in cytotoxicity of H_2S and H_2Se have shown H_2Se to be more toxic. Hence, the authors attribute the same reasons in this study too. The presence of Se makes the TMD (WSe_2) more toxic than the others.[260] The same group also studied the toxicity of layered GaS and GaSe. The authors found that GaSe is more toxic than other commonly known transition metal chalcogenides. The difference in the cytotoxicity is attributed to the high solubility of the Ga ions in the cellular environmental pH.[130] The use of nanomaterials for different potential applications such as drug delivery requires cytotoxicity assessment.[386−388] Dhenadhayalan et al. reported the use of 2D nanosheets of MoO_3, MoS_2 and $MoSe_2$ as biomarkers for prostate cancer. The viability measurements were studied using human embryonic kidney 293T (HEK) cells. The cells were exposed with different dosage of nanosheets (up to 100 μg/mL) for 48 h; the results showed an 80% viability.[389] In another study, lipid functionalized WS_2 and MoS_2 sheets were studied for drug delivery application. The van der Walls force helped in the functionalization of liposomes, and the drug loading was enabled with hydrogen bonding. HeLa cells exposed with different dosage of nanosheets concentration exhibited nontoxic attributes up to 50 μg/mL using MTT assay.[390]

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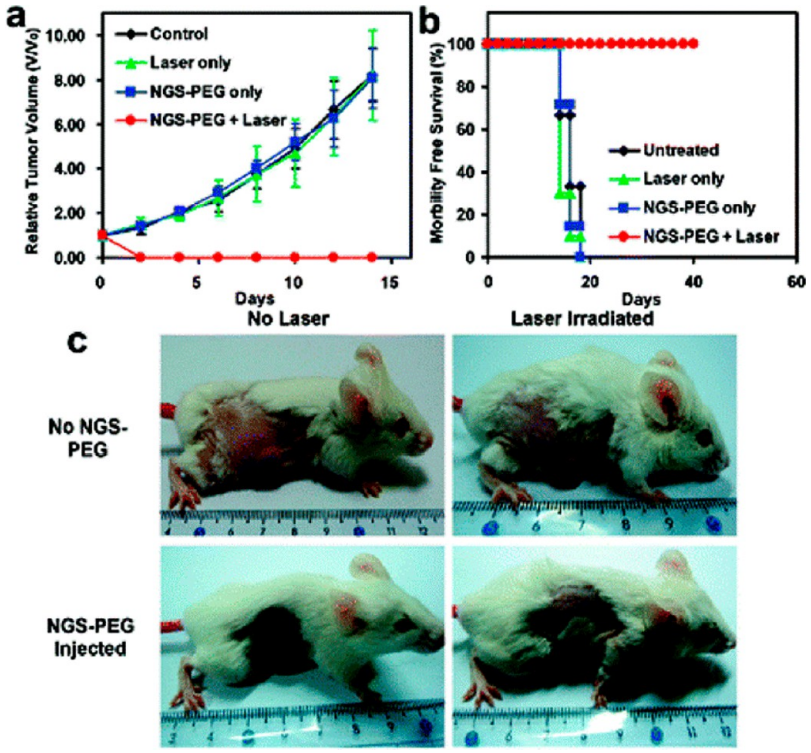

Figure 29. In vivo photothermal therapy study using intravenously injected NGS−PEG. (a) Tumor growth curves of different groups after treatment. While injection of NGS−PEG by itself or laser irradiation on uninjected mice did not affect tumor growth, tumors in the treated group were completely eliminated after NGS−PEG injection and the followed NIR laser irradiation. (b) Survival curves of mice bearing 4T1 tumor after various treatments are indicated. NGS−PEG injected mice after photothermal therapy survived over 40 days without any single death. (c) Representative photos of tumors on mice after various treatments are indicated. The laser irradiated tumor on the NGS injected mouse was completely destroyed. Reproduced with permission from ref 330. Copyright 2010 American Chemical Society.[380]

This proved the biocompatibility of the nanosheets. Li et al. studied the potential applications of nanodiamonds for drug delivery. The toxicity studies revealed that the presence of serum protein in the cell culture medium induced acute levels of toxicity.[391] In a similar attempt Prylutska et al. investigated the utilization of fullerene NPs for drug delivery application. The fullerene-doxorubicin complex showed a small variation in toxicity compared to the bare C_{60} NPs. The toxicity effect of functionalized fullerene nanoparticles was also evaluated C_{60} NPs were covalently functionalized with PEG of different sizes. All the NPs hindered the metabolic activity but the fullerene NPs with longer chains of PEG showing less order of toxicity, and hence, the magnitude of toxicity followed the order of decreasing chain size of PEG.[392] In another study, the authors explored the use of fullerene NPs for pH sensitive drug delivery in tumor-bearing mice. The C_{60} NPs are functionalized/derivated and were extremely pH sensitive. The derivated C_{60} molecules showed efficient tumor targeting capability and released 2.4 times greater drug deposition to the tumor cells than the normal cells.[393] Similarly, a novel composite of FePt@CoS_2, yolk−shell nanocrystals for drug anticancer medicine. The as-prepared samples exhibited high level of toxicity on being exposed to human cervical carcinoma (HeLa) cell lines.[394] Similar reports of therapeutic applications were reported recently. Hollow ZrO_2/polypyrrole (PPy) was used for improved drug delivery and real-time CT monitoring in synergistic photothermal-chemo cancer therapy. In this study the following composite and the zirconia NPs did not show any potential levels of toxicity. After being exposed up to 72 h the cells showed impressive biocompatibility toward the NPs.[395]

Amidst all other nanomaterials, quantum dots have acquired a reasonable attention of being biocompatible and offers sufficient scope worth exploration for various biomedical applications such as drug carrier, cellular imaging etc. In a study, the authors aim to understand the cytotoxicity and the cellular uptake mechanism of chitosan capped Bi_2S_3 quantum dots. It was observed that the quantum dots were internalized using endocytic pathway and they were nontoxic in nature. Capping the NPs with chitosan resulted in the formation of biocompatible quantum dots.[396] In another study, the authors prepared aspirin-based carbon dots and evaluated its toxic potential. The composites were evaluated on mouse leukemic monocyte macrophage (RAW264.7), HeLa, human mouth epidermal carcinoma (KB), and bone marrow stromal cells (BMSC) cell lines with dosage up to 100 μg/mL and exposed for 24 h. The cytotoxicity was further evaluated using confocal laser scanning microscopy, hematology, and serum biochemistry. The results suggested that the quantum dots were nontoxic in nature and can readily serve bifunctional applications such as anti-inflammatory and cellular imaging agents.[397] Huang et al. studied the in vivo kinetic behavior of the carbon dots and their fate. C-dots are efficiently and rapidly excreted from the body after all three injection routes. The clearance rate of C-dots is ranked as intravenous > intramuscular > subcutaneous.[398] Fasbender et al. evaluated the uptake dynamics and the toxicity of the graphene quantum dots. The quantum dots were evaluated on human leukocytes cell lines with dosages up to 500 μg/mL and exposed for 36 h. The NPs were internalized via phagocytosis and the uptake was concentration dependent and moreover, the quantum dots showed very low

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levels of toxicity.[399] Su et al. and Chen and co-workers in two independent studies investigated the toxic influence of CdTe quantum dots. In both studies, the authors clarified that toxicity is observed due to the leaching of cadmium ions from the surface of the crystal lattice (Figure 30). Cell death was

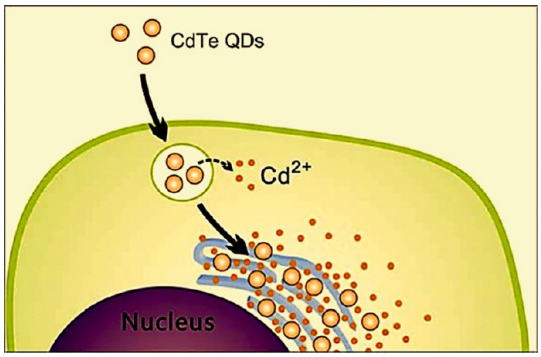

Figure 30. Illustration of cytotoxicity induced by cadmium-based QDs. Reproduced with permission from ref 155. Copyright 2012 Royal Society of Chemistry.[155]

observed due to the restraint of metabolic activities rather from the direct cell death induced by Quantum dots. The cadmium ions instigated oxidative stress in the cells and decreased the metabolic activity.[155,400,401]

Ambrosone et al. investigated the mechanism responsible for toxicity induced by CdTe quantum dots in Hydra vulgaris using BrdU assay, TUNNEL technique, and caspase assay. The sublethal doses of QDs caused time and dose dependent morphological damages more severe than Cd^{2+} ions at the same concentrations, which impaired both reproductive and regenerative capability and activated biochemical and molecular responses.[402] Chen et al. reported a novel MXene quantum dot of Ti_3C_2 for intracellular pH sensor. The potential use of these quantum dots as fluorescent sensor and to ensure the biocompatibility of them, the in vitro cytotoxicity was evaluated. MCF-7 cells were exposed for 24 h by different concentrations of the quantum dots, and later the toxicity estimation was done using MTT assay. The cellular viability appeared to be more than 80%, which showed the low toxicity and the biocompatibility of the quantum dots.[403] In another new report, the authors reported the fabrication of a novel composite of graphene oxide conjugated with folic acid and gadolinium-labeled dendrimer (FA-GCGLD). The as-prepared composite illustrated impressive possibility of magnetic resonance imaging-guided combined chemotherapy. The in vivo studies showed a quick accumulation of tumor cells, which facilitated the systemic distribution of particles and also inhibited tumor growth. HepG2 and HeLa cells were utilized to evaluate the cytotoxicity using CCK-8 assay. The cells were exposed for 48 h and the viability results indicated no significant change, which further exhibited the biocompatibility of the composite developed.[404] Spangler et al. reported the synthesis of $CuInS_2/ZnS$ core/shell NPs. The authors studied the applicability of the quantum dots for bioimaging and further the biocompatibility of the dots was evaluated. THP-1 cells exposed for 6 h showed negligible reduction in the viability measurements.[405] Studies illustrating the toxic profile of other new generation 2D materials such as graphitic carbon nitride against bacterial strains are reported widely.[406,407] The microbial inactivation utilizing C_3N_4 was first investigated by Wang et al. The authors studied the bacterial inactivation of *E. coli* K12, using heterojunction photocatalyst of α-sulfur comodified with graphene and graphitic carbon nitrides. Two different structures were fabricated by altering the arrangement of sheets of carbon nitride ($CNRGOS_8$) or the reduced graphene ($RGOCNS_8$) across the α-sulfur. The $CNRGOS_8$ showed higher photocatalytic disinfection efficiency compared to $RGOCNS_8$. Moreover, the core−shell composite particles displayed reduced photocatalytic disinfection in anaerobic condition. The photogenerated electrons react directly on the

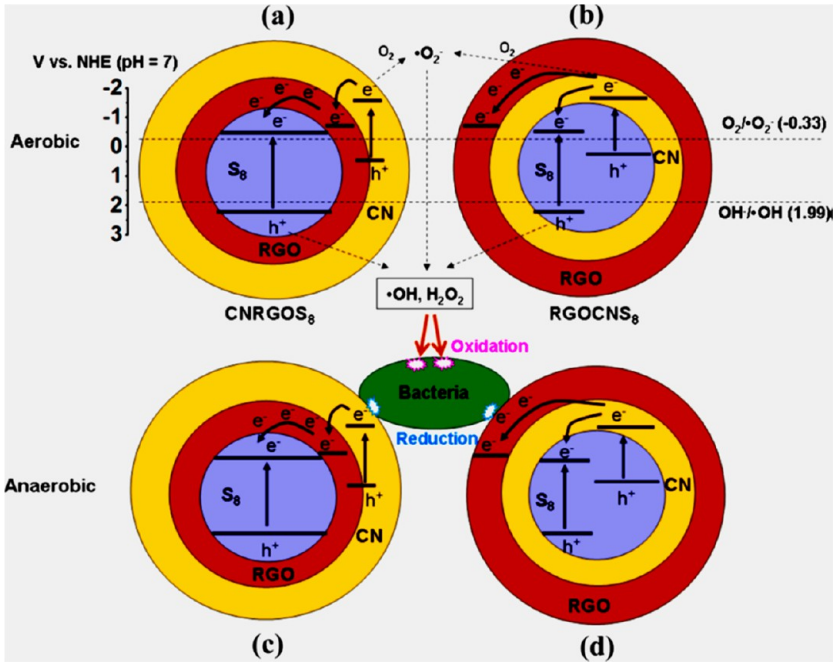

Figure 31. Disinfection mechanism of the composite structures in the aerobic and anaerobic condition. Reproduced with permission from ref 408. Copyright 2013 American Chemical Society.[408]

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Table 1. Global Strategies to Address Human Health and/or Environmental Safety Aspects of Nanomaterials[a]

organization	objectives
The Organisation for Economic Co-operation and Development (OECD)	Evaluation of risk assessment approaches for manufactured nanomaterials through information exchange and the identification of opportunities to strengthen and enhance risk assessment capacity.
National Institute for Occupational Safety and Health (NIOSH)	Development and implementation of commercial nanotechnology through conducting strategic planning and research to provide national and world leadership for incorporating research findings about the implications and applications of nanotechnology into good occupational safety and health practice.
EU NanoSafetyCluster	Works on the projects addressing all aspects of nanosafety including toxicology, ecotoxicology, exposure assessment, mechanisms of interaction, risk assessment, and standardization.
	Conducts workshops and seminars to educate people, particularly all nanotechnology workers.
Federal Institute for Materials Research and Testing (BAM)	BAM is involved in EU-funded (FP7) projects: NanoDefine (FP7): The aim of NanoDefine is to support the governance challenges associated with the implementation of the nanomaterial legislation by addressing the issues on the availability of suitable measuring techniques, reference material, validated methods, acceptable for all stakeholders and delivering an integrated and interdisciplinary approach.
	NanoValid (FP7): The main objective of NanoValid is the development of new reference methods and certified reference materials, including methods for characterization, detection/quantification, dispersion control and labeling, as well as hazard identification, exposure, and risk assessment of ENs.
Federal Ministry of Education and Research (BMBF)	NanoCare: safe handling of manufactured nanomaterials.
	Studying the effects of ENM on humans and the environment.
Federal Environment Agency (Umweltbundesamt, UBA)	NanoCare: safe handling of manufactured nanomaterials.
	Investigating impacts on health and the environment.
Federal Institute for Risk Assessment (Bundesinstitut für Risikobewertung, BfR)	To demonstrate and establish new principles and ideas based on data from value chain implementation studies.
	To establish safe-by-design as a fundamental pillar in the validation of a novel manufactured nanomaterial.
	Establishing nanomaterial grouping/classification strategies according to toxicity and biological effects for supporting risk assessment.
	Grouping of nanostructured materials for protection of workers, consumers, the environment and risk minimization.
Federal Institute of Occupational Safety and Health (BAuA)	Grouping of nanostructured materials for protection of workers, consumers, the environment, and risk minimization.
Federal Research Institute of Nutrition and Food (Max Rubner-Institut, MRI)	Detection and characterization of nanomaterials in complex matrixes such as food.
	Research on nanosized carrier systems for bioactive compounds.
	Interaction of nanomaterials with compounds of the food matrix.
Modena Cost	The specific objectives are to study the synthesis of engineered nanomaterials (ENM) with controlled composition, size, area and nanotexture and develop strategies to immobilize ENM in matrices, on substrates with minimum effect on the desired properties and surface reactivity and identify the relevant data sets for quantitative nanostructure−toxicity relationships (QNTR) modeling.

[a]Reprinted with permission of Viswanath et al. (2016). Full details are given in the respective publication.[414].

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Table 2. Examples of Various Kinds of Nanoscale Materials That Might Be Present within Foods and Their Origin[a]

nanoscale material	origin	characteristics	products
		organic nanoparticles	
casein micelles	natural	protein−mineral clusters	milk, cream
cell organelles	natural	ribosomes, vacuoles, lysosomes, etc.	meat, fish, fruits, vegetables, spices
oil bodies	natural	phospholipid/protein-coated triglyceride droplets	plants, seeds
lipid nanoparticles	ENP	solid particles or liquid droplets coated by emulsifiers	some beverages, sauces, dressings, creams
protein nanoparticles	ENP	clusters of protein molecules held together by physical or covalent interactions	mainly in development
carbohydrate nanoparticles	ENP	small solid fragments extracted from starch, cellulose, or chitosan; clusters of polysaccharide molecules held together by physical or covalent interactions	mainly in development
		inorganic nanoparticles	
iron oxide	ENP	FeO nanoparticles used to fortify foods with iron	nutritional supplements, sausage casings
titanium dioxide	ENP	TiO_2 nanoparticles used as whitening agents	candies, chewing gums, bakery goods, milk powders
silicon dioxide	ENP	SiO_2 nanoparticles used to control powder flowability	salts, icing sugar, spices, dried milk, and dry mixes
silver	ENP	Ag nanoparticles used as antimicrobials in foods, coatings and packaging	meat, food packages, containers, coatings

[a]Reprinted with the permission of McClements et al. (2017). Full details are given in the respective publication.[417].

bacteria to cause inactivation. The disinfection mechanism of the composite structures in the aerobic and anaerobic condition is illustrated in Figure 31.[408]

Safety Guidelines for Handling Nanomaterials. The toxicological profile of nanomaterials is an evolving chapter, as studies on new properties and their toxicity impact is uncertain.[409,410] Guidelines for safe and protective handling of nanomaterials for institutes and industries have been adapted in recent years.[411,412] These guidelines do not provide exact steps to circumvent the problem, but they definitely are rules for good practice to avoid potential risk.[413] The basic framework for all these guidelines is outlined below:

1. Assessing risks and identifying uncertainties in the fabrication process and use of nanomaterials.
2. Elaborating and employing an effective approach to address and control the risks.
3. Prevention and control of the necessary exposure.
4. Warranting the follow up of the implemented process and implementing necessary measures toward it.
5. Examining the exposure levels and undertaking proper surveillance.
6. Initiating adequate health analysis.
7. Steps and protocols to be employed in advance against any accidents or emergency.
8. Adequate training, informing, and supervision of the students or employees of the institution and industries, respectively.

Apart from these guidelines, there is a serious discussion among the academic and the policy makers to address the grave threat posed by nanomaterials. A recent book chapter highlighted the global actions taken in regards to the health and safety concerns (Table 1).[414]

The use of engineered nanomaterials in daily products has necessarily improved our quality of life, but at the same time, it has posed a concern among regulatory agencies and academic researchers about their unknown adverse effects. The use of engineered NMs has grown exponentially in the past decade, but it concerns policy makers and regulatory keepers. There exists a plausible chance of unknown materials creeping into the consumer market even without the knowledge of the authorities. Household products such as food, personal care items, and several such other products are on the watchdog of the regulatory authorities and environmental advocacy groups.[415,416]

A recent review highlights the nature of toxicity of the nanomaterials posed in our food products. A list of organic and inorganic NMs used vividly in commercial products is provided in Table 2.[417]

CONCLUSIONS AND OUTLOOK

In this review, we attempted to provide a comprehensive outlook of nanotoxicity and its various aspects. In retrospect, despite the presence of several studies and reviews in literature, there exists a definite paucity in complete understanding of toxicity and its impact on the human world. As discussed earlier, the toxicity of the nanomaterials depends on several physicochemical properties, and alteration of any single parameter will impact the toxicity pattern and result in a different physiological end point. A lack of correlation between in vitro, in vivo, and in silico data is witnessed in different nanomaterials. Thus, the need for toxicity libraries of nanomaterials has grown in the past few decades. Combining the different toxicity assaying techniques and forming a hub for underlining the potential toxicity of the materials can prevent as well as help to predict toxicity of several newly engineered nanomaterials. Hence, there is a requirement for extensive studies to match the scale of different nanomaterials produced and utilized in large measure in industrial applications. This will also aid in the growth of our fundamental understanding of toxicology and its impact on the environment and humans.

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Notes

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ABBREVIATIONS USED

NMs, nanomaterials
HDF, human dermal fibroblasts
TMD, transition metal dichalcogenide
Ag, silver
BBB, blood brain barrier
MWCNT, multiwalled carbon nanotube
NADPH, nicotinamide adenine dinucleotide phosphate
PVP, polyvinylpyrollidone
NLS, nuclear localization signal
CTAB, cetyltrimethylammonium bromide
PAA, polyacrylic acid
NIR, near-infrared
PEG, polyethylene glycol
SPIONS, superparamagnetic iron oxide nanoparticles
PLGA, poly(lactic-co-glycolic acid)
ALD, atomic layer deposition
CNT, carbon nanotube
SEM, scanning electron microscopy
TMD, transition metal dichalcogenide
CNT, carbon nanotube
ALD, atomic layer deposition
BBB, blood brain barrier
QNTR, quantitative nanostructure−toxicity relationships
HDF, human dermal fibroblasts
BEAS, human bronchial epithelial cells
K17, human keratinocytes
TEM, transmission electron microscopy
NADPH, nicotinamide adenine dinucleotide phosphate
Au, gold
si-RNA, small interference ribo nucleic acid
MAPK, mitogen-activated protein kinases
ROS, reactive oxygen species
ED_{50}, effective dose
LD_{50}, lethal dose
DDT, dichlorodiphenyltrichloroethane
NPs, nanoparticles
NNI, National Nanotechnology Initiative
NADPH, nicotinamide adenine dinucleotide phosphate
TB, trypan blue
LDH, lactate dehydrogenase
ICP-MS, inductively coupled plasma-mass spectrometry
DMSO, dimethyl sulfoxide
CNS, central nervous system
RES, reticuloendothelial system
NGS, nano graphene sheets
PEI, polyethylenimine
XAS, X-ray absorption spectroscopy
EDX, energy-dispersive X-ray spectroscopy

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