

Evaluating Nanotoxicity: Integrating *In vitro* and *In vivo* Models for Risk Assessment

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Nanotechnology, involving the manipulation of materials at the nanoscale, has vast applications in healthcare, including drug delivery, gene therapy, and cancer treatment. Nanomaterials (NM) such as nanoparticles (NP's) and nanotubes exhibit unique properties that raise concerns about potential toxicity. Nanotoxicology studies the safety of these materials, focusing on their physicochemical properties and toxicological effects like genotoxicity and cellular damage. *In vitro* methods, including cytotoxicity, apoptosis, and genotoxicity assays, provide controlled environments for testing nanoparticle interactions with biological systems. *In vivo* methods further assess the effects in living organisms, including biodistribution and histopathological changes. New technologies, such as high-throughput screening, Nano-QSAR (quantitative structure -activity relationship) computational models, and stem cell-based assays, are enhancing toxicity prediction. Omics technologies (e.g., genomics and proteomics) offer a comprehensive understanding of how nanoparticles affect biological systems. These advanced techniques are crucial for ensuring the safe development and use of nanomaterials.

Keywords: Nanotoxicology, Nanoparticles, Nanotechnology, Genotoxicity, *In vitro* methods.

The prefix “nano” originates from the Greek word “nanos,” which means “dwarf.” Nanotechnology is the manipulation and application of artificial particles or systems that have at least one dimension smaller than 100 nanometers (nm). A structure that has at least one size (length, height, and breadth) smaller than 100 nanometers (10^{-7} meters) and as huge as a virus particle is designated as a nanomaterial. They are categorized depending on their size, dimensions (0, 1, 2, 3D), composition, shape (nanoparticle, nanotube, nanostick, nanofiber etc.), source (natural, synthetic), and content (carbon-derived, composite-based organic-based,

etc.). The most often used types of nanomaterials are nanoparticles, which have three dimensions that are all equal and smaller than 100 nanometers.¹ “Nanoparticles” that are found naturally or as results of other operations, such as carbon black, fire smoke, or welding fumes; rather, the word refers solely to manmade particles (such as nanotubes made of carbon, iron oxides, fullerenes, etc.² Humans are exposed to nanoparticles both directly and indirectly as a result of their growing use in commercial applications such as semiconductor components, skincare products, water filtration, fillers, opacifiers, catalysts, and

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microelectronics.³ In order to target certain cells, biomolecules like DNA, proteins, and monoclonal antibodies are frequently purposefully coated onto nanomaterials for imaging and drug delivery.⁴ When it comes to their use in commercial items, industrial processes, and biology, nanomaterials have had a significant impact. This technology, known as nanotechnology, has the potential to bring about a new revolution in society.⁵

Therapeutic applications of Nanoparticles a relatively recent subject of the health sciences, is one of the emerging fields. Consequently, a number of nanoparticles are being investigated or employed in several clinical domains, such as therapy, smart ways to deliver drugs, surgery, medical implants, treatment of illnesses or cancers, and gene delivery as mentioned in the Figure 1.

Gene therapy

The intention of gene therapy, a subject that is extensively researched, is to fix malfunctioning genes in order to prevent and treat genetic illnesses.

Antibacterial activity

Multiple silver nanoparticles have been demonstrated to possess antibacterial activity; these can also be used alongside with other drugs for reducing antibiotic resistance.

ZnO(Zinc oxide) nanoparticles may be regarded as a useful adjuvant in ciprofloxacin combination therapy due to their possible synergistic action with the antibiotic.⁶

Cancer diagnosis and treatment

Carbon nanotubes are employed in the identification of biomarkers and the disclosure of DNA alterations.⁷ Quantum dots are used with magnetic resonance imaging to more precisely reveal tumor location.⁸

Neurological impairment therapy

Applications of nanotechnologies show promise in the management of the neurological disorder and the repair of damaged axons. Utilizing nanoparticles with a strong affinity for circulating amyloid- β with an emphasis on protecting neuronal tissue.⁹

Orthopedic implants

Collagen, hyaluronic acid, chitosan, and titanium alloys are examples of natural and synthetic polymers that are frequently utilized as nanomaterials in bone and cartilage tissue engineering.¹⁰

Therapies for skin health

Novel nanoscale materials that have been created and produced to solve current wound care issues. Drug delivery, growth factor supplements, hydrogels, biodendrimers, electrospun nanofibers, and other polymer therapeutic conjugates have all been employed as skin substitutes in wound healing procedures.¹¹

Nanotoxicity

A novel area of toxicology called nanotoxicology was suggested to fill in the information gaps and to explicitly address the potential negative health effects of nanomaterials. Nanomaterials are being used more and more in industrial applications, consumer goods, and medical devices as nanotechnology develops quickly. Their distinct characteristics, like size, surface area, and reactivity, can, nevertheless, result in unanticipated toxicological effects that are not found with bulk materials. Nanotoxicology encompasses the domains of exposure pathways, molecular determinants, physicochemical factors, biodistribution & genotoxicity.¹²

Physicochemical properties of nanoparticles

Nanoparticles possess unique physicochemical properties that distinguish them from their bulk counterparts, such as extremely small size and a high surface area-to-volume ratio as shown in the Figure 2. These features grant them enhanced reactivity, improved cellular uptake. However, the same properties that provide

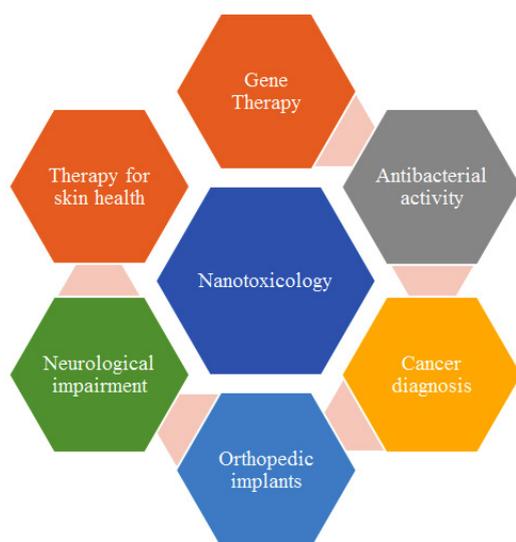


Fig. 1. Therapeutic applications of Nanoparticles

functional advantages can also introduce novel mechanisms of toxicity. Their small size allows nanoparticles to interact with cellular structures at the molecular level, potentially disrupting normal biological functions. High surface reactivity can lead to the generation of reactive oxygen species (ROS), causing oxidative stress, inflammation, and cellular damage. Different shapes and morphologies, such as rods or fibers, may lead to mechanical damage or hinder cellular clearance, prolonging exposure. Thus, nanoparticle-induced toxicity arises not from a single factor but from a complex interplay of size, surface area, composition, shape, and other physicochemical traits.

Particle size and surface area

Materials' surface area seems to grow exponentially in proportion to their volume as they get smaller, which raises the nanomaterial's surface reactivity to its surroundings and to itself. Notably, particle size and surface area affect how the system responds to, distributes, and eliminates the materials.¹⁴ To evaluate the *in vitro* cytotoxicity of NPs of different sizes, several groups have employed a variety of cell types, incubation conditions, and duration of exposure.^{15,16} One of the primary reasons for the toxicity of ENMs (Engineered Nanomaterials) *in vivo* is the generation of reactive oxygen species through the creation of free radicals. Dimensions

has significance in this process because, as many writers have observed, the smaller the object, the more likely it is to generate ROS (Reactive oxygen species).¹⁷ Numerous investigations utilizing a range of nanoparticle classes demonstrated that surface area plays a key role in exhibiting hazardous effects.¹⁸ While NPs larger than 50 nm do not spread to other tissues and are readily absorbed by RES (Reticulo endothelial system), NPs smaller than 50 nm have been found to traverse rapidly to almost all tissues and produce possibly harmful symptoms in a variety of tissues.¹⁹

Effect of Particle Shape

Numerous nanoparticles, such as silica, allotropies, carbon nanotubes, gold, nickel and titanium nanomaterials have been shown to have shape-dependent toxicity.²⁰⁻²³ In contrast to nanoparticles that are fibre-like or rod-shaped nanoparticles, spherical nanoparticles have been found to undergo endocytosis more easily and quickly.²⁴ Likewise, it was demonstrated that the absorption of gold nanorods is not as quick as spherical nanospheres and that it peaks when the aspect ratio is close to unity.²⁵

Effect of Surface Charge

Surface charge has a substantial impact on nanoparticle toxicity as well. Because of their improved opsonization by the plasma proteins, positively charged nanoparticles exhibit notable cellular absorption in contrast to negatively charged

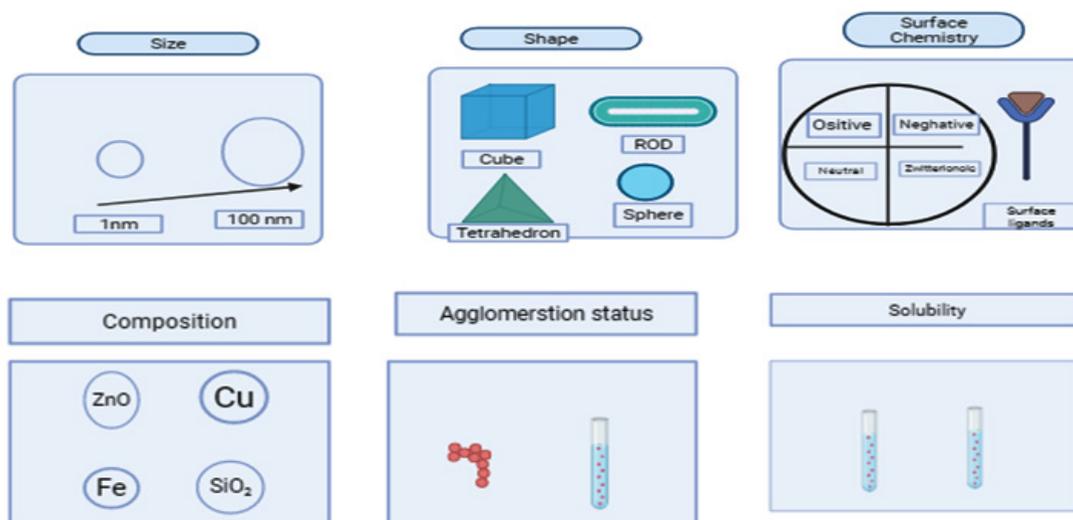


Fig. 2. Physicochemical properties affecting metal based nanoparticle toxicity-Different sizes and shapes. Created in <https://BioRender.com>

and neutral nanoparticles. Additionally, they have been demonstrated to cause platelet aggregation and hemolysis,²⁶ besides it was also noted that the surface charge of nanoparticles modifies the transmembrane permeability and blood-brain barrier integrity.

Effect of Aggregation and Concentration

Research findings demonstrated that well-dispersed carbon nanotubes had cytotoxic effects when contrasted with those of conventionally purified rope-like agglomerated carbon nanotubes and asbestos as a reference.²⁷

Routes of exposures

One definition of “exposure” is the potential for an agent or substance from the outside environment, such as a nanoparticle (NP), to enter the body as shown in the Figure 3. Furthermore, the potential toxicity is determined by the exposure route.²⁸

Inhalation Exposures

Exposures through inhalation are known to occur largely in professional environments, have been the focus of a large portion of exposure and toxicological research efforts and measurements using NPs to date. In fact, according to research on animals, the majority of the harmful effects of inhaled NPs or particles affect the cellular components in the anatomical compartment of the respiratory tract, namely at the portal entrance sites. The lungs capillaries are where carbon dioxide and oxygen exchange gases. Although the possibility is thought to be minimal or infrequent, it is possible that inhaled NPs could eventually enter the systemic circulation through this pathway.²⁹

Dermal Exposure

In essence, the skin is made up of two main sections: the larger, known as the epidermis, and the dermal component, which is located

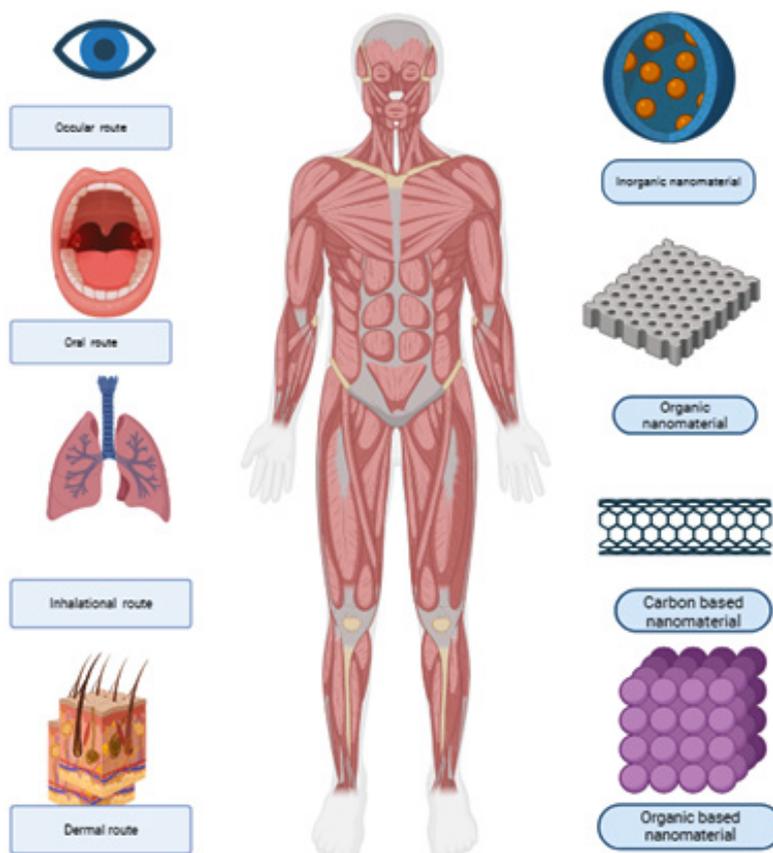


Fig. 3. Different routes of exposure of nanoparticles. Created in <https://BioRender.com>

Table 1. An overview of the research on nanomaterial toxicity tests

Test Method	Purpose	Nanomaterial Tested	Outcomes	Year	References
Light Microscopy	Characteristics of physicochemistry	Singled walled carbon nanotubes, silver particles	Observes the morphology and size of nanomaterials, assessing their properties.	2006	58, 59
Hemoglobin Estimation	Haemolysis	SiO ₂	Assesses red blood cell lysis due to exposure to nanomaterials	2011	60
Comet Assay	DNA damage	Metal, Metal oxide nanoparticles	Quantifies the amount of DNA damage (strand breaks) in cells that have been exposed to nanoparticles.	2010	61
Micronucleus test	Genotoxicity	Different types of nanoparticles	Detects DNA damage and chromosomal aberrations caused by nanoparticles.	2011	62
Lactate Dehydrogenase (LDH) Assay	Cell viability	Carbon nanoparticles	Assesses cell membrane integrity and cytotoxicity.	2005	63, 64
Tetrazolium Salts (MTT, MTS, etc.) Assay	Cell viability	Carbon nanoparticles, Fullerenes	Measures metabolic activity of cells to assess cytotoxicity.	2005; 2004	65, 66
Alamar Blue Assay	Cell viability	Quantum dots	Detects metabolic activity in cells to evaluate potential toxicity.	2006	67
Caspase-3 Activity Assay	Apoptosis	Silver Nanoparticles	Measures apoptotic cell death by detecting activated caspase-3.	2009	58,59
Lipid Peroxidation, Vitamin E Assay	Oxidative stress	Single-walled carbon nanotubes	Measures lipid peroxidation & antioxidant defense in cells.	2012	68
Reactive Oxygen Species (ROS) Production	Oxidative stress	Oxidative stress	Measures oxidative stress by quantifying ROS production in cells.	2011	69

underneath the epidermis and has a restricted supply of blood vessels. Even after prolonged application, Lademann noted that no particles were seen in the stratum corneum's deeper levels.³⁰ Exposures via the gastrointestinal tract happen after consuming food or after swallowing and pulmonary clearance of inhaled nanoparticles.³¹

Oral or Ingestion Exposures

More precisely, food coloring, supplements, and flavor enhancers are a few examples of ingested component sources.³² Depending on the kind of nanoparticle and its characteristics, the possibility that it will pass through the digestive system varies; many are simply expelled without entering the body. According to some researchers, oral exposure to NPs may cause absorption through the gut-associated lymphoid tissue's Peyer's patches' epithelial cells. On an average, micro-copper particles do not cause significant harm to the liver, kidney or spleen of experimental mice but nanoparticles do. The findings point to a gender-dependent aspect of nanotoxicity. The findings point to a gender-dependent aspect of nanotoxicity.³³ According to recent research,³⁴ NPs administered orally may be absorbed through the lymph nodes and travel throughout the gastrointestinal tract.

Biodistribution of Nanoparticles

Physical clearance processes (such as epithelial endocytosis, mucociliary movement, lymphatic drainage, interstitial translocation & blood circulation,) and chemical clearance processes (such as leaching, protein binding & dissolution) are implicated in the studies conducted thus far. Certain nanoparticles may build up in the liver as a result of first-pass metabolism.³⁵ Nanoparticles are transferred to the bone marrow, colon, lungs, spleen, liver and lymphatics following intravenous injection.³⁶ Differences in rate of clearance & confinement of nanoparticles by macrophages are a result of differential opsonization.³⁷ Opsonization events must be suppressed at certain locations or anatomical compartments in order to enhance the non-invasive retention of nanoparticles in the bloodstream. For instance, a poly(ethylene) glycol (PEG) coating would make hydrophobic particles more hydrophilic, lengthening the time it takes for them to circulate throughout the body.³⁸

Genotoxicity

The genotoxic potential of NM exposure

is a major worry. The ability of physical or chemical substances to change genetic information is known as genotoxicity. Genotoxic events can result in permanent alterations (mutations) in the quantity or structure of a cell's genetic material, or they might be temporary (repairable harm). A genetic condition may result from a mutation in a germ cell that is passed down to the following generation. Cancer in somatic cells can result from a mutation in a crucial gene. Therefore, all mutagens are thought to have the potential to cause cancer.³⁹ Several studies have been conducted on the genotoxicity of NMs and the processes that result in temporary or permanent genetic alterations. According to recent research, there are two primary (direct or indirect) and secondary genotoxicity mechanisms that might cause NM genotoxicity.^{40,41} Physical contact between NMs and DNA in the nucleus is necessary for primary direct genotoxicity, which is brought on by direct interaction between NMs and the genome. Large DNA malformations, chromosomal damage, and DNA breaks and other diseases could result from this interaction. Physical contact between NMs and DNA in the nucleus is necessary for primary direct genotoxicity, which is brought on by direct interaction between NMs and the genome. Large DNA malformations, chromosomal damage, and DNA breaks and other diseases could result from this interaction. The main indirect mechanism is caused by reactive oxygen species (ROS) triggered by NMs or by toxic ions released during NM dissolution and intracellular ROS formation through the Fenton-type reaction.^{42,43} The primary mechanism of NM genotoxicity is thought to be secondary genotoxicity. ROS generated by inflammatory cells mediate it. By activating phagocytes, NMs can generate an oxidative burst. This inflammation, which aids in clearance, is the body's first line of defense against the invasion of microbes or other objects.⁴⁴ A range of tests covering DNA damage, gene mutations, and chromosomal damage as sensitive genotoxicity endpoints are included in the category of genotoxicity biomarkers. The number of NMs produced is constantly increasing despite the lack of information and limited data regarding NM safety. For this reason, attempts have been made to comprehend how NMs work and to create or modify approved OECD testing procedures for chemicals for use on NMs in

a number of previous and ongoing European projects, including NanoGENOTOX, NanoTEST, NanoReg, RiskGONE, HISENTS, RiskGONE, and others.⁴⁵ There are issues with the mode of action (MoA), specificity and data of regulatory *In vitro* genotoxicity testing.⁴⁶

Methods for assessing toxicity of nanomaterials

***In Vitro* Methods**

They are essential to nanotoxicology because they offer a controlled setting for examining interactions of nanoparticles with biological systems, including human cells, tissues, and other model species. *In vitro* toxicity studies often look at the following parameters: oxidative stress, inflammatory alterations, apoptosis, DNA mutation/damage and cytotoxicity. Inflammatory alterations are an additional parameter that can be assessed using *in vitro* testing. Inflammatory indicators can be found using the enzyme-linked immunosorbent test (ELISA).⁴⁷ Moreover, *in vitro* tests can also be used to assess cytotoxicity or necrosis. Assays for cytotoxicity include lactate dehydrogenase (LDH), clonogenic assays, Trypan blue assays, Tetrazolium salts assays, and Alamar blue assays.⁴⁸

Proliferation Assay

One important factor in the growth and progression of tumors is cell proliferation, which can also represent the surrounding cells' compensating reaction to necrosis or apoptosis. Currently, there are several well-established techniques for measuring cell proliferation each with unique limitations and specificity. Currently used techniques include flow cytometry, immunohistochemistry, and histochemistry. Histochemical techniques include the absorption of the DNA analog bromodeoxyuridine (BrdU), incorporation of tritiated thymidine ([³H] thymidine), and direct detection of mitosis by staining for DNA content⁴⁸. In immunohistochemistry, Ki-67 and antibodies that identify proliferating cell nuclear antigens (PCNAs) are now of interest.⁴⁹

Apoptosis Assay

This assay assays the activity of caspase-3 and caspase-7 using luminescence. Cell lysis and substrate cleavage by caspase occur when caspase reagent is added to NP-treated cells. Luciferase then generates a luminous signal that is equivalent to the amount of caspase activity that is available.⁵⁰

Genotoxicity Assays

Chromosomal aberration induction

This method involves assessing how NPs affect the quantity of cells and alterations in the physical appearance of chromosomes. Cells are treated with NPs during the S-phase because they are more responsive during this phase. Colcemid or colchicine is then administered at predetermined intervals. Trypsinated cells are counted after chromosomes are processed and stained with Giemsa stain for microscopic evaluation of aberration (chromatid exchange, chromatid breaks, chromosomal breaks, chromosome fragmentation)⁵¹ It is employed to measure and evaluate cellular DNA damage. After cells have been embedded in a thin agarose gel and put on a microscope slide, the cells are lysed to extract the cellular proteins. In neutral or alkaline circumstances, allowing the DNA to uncoil before being subjected to electrophoresis, which allows the pieces of DNA to detach from the nucleus. The amount of fluorescence in the head and tail length is determined by staining with either propidium iodide or ethidium bromide. The amount of damaged DNA is directly equivalent to the amount of DNA released from the comet's head.^{52,53}

Measurement of oxidized guanine bases

Single base changes within a specific gene can be detected by assaying any one of the many oxidized guanine bases, including 7,8-dihydro-oxodeoxyguanine (oxo-dG) and 8-hydroxydeoxyguanosine (8-OHdG). These bases alterations are often caused by oxidative damage and are quantified by HPLC or immunohistochemistry.⁵⁴

***In Vivo* Methods**

Using animal models, such as mice and rats. *In situ* toxicity is typically assessed. The methods used to evaluate *in vivo* toxicity include histopathology, biological distribution, clearance, hemophilia, and plasma chemistry. Biodistribution studies examine how nanoparticles enter organs or tissues. Nanoparticles can be detected in living and expired organisms using radiolabels.⁵⁵ The liver, kidneys, heart, brain and spleen are among the tissues exposed to nanoparticles, according to histopathology research.⁵⁶ Examining alterations in cell type and serum chemistry following nanoparticle exposure is an additional method of determining *in vivo* toxicity.⁵⁷

Novel Technologies Employed For Evaluation Of Toxicity

Ever since the development of nanotechnology, a growing number of chemicals have entered the environment, necessitating the collection of data regarding their toxicity. Traditional toxicity assessment using animal models is often not practical in these circumstances due to its poor capacity, time-consuming nature, high expense, and limited evaluation of endpoints. The authors claim that this method, in conjunction with functional toxicogenomics, can be crucial in determining the key biological elements and pathways implicated in the toxicity response.⁷⁰

Genome arrays have been employed to evaluate the impact of nanoparticles. The expression of many genes linked to neurodegenerative illness, immune cell function and motor neuron disorders was altered by the inhaled silver nanoparticles, suggesting possible immunotoxicity and neurotoxicity.⁷¹ The use of high-throughput screening (HTS) techniques to check how silver nanoparticles affect bacterial cells is said to aid in tracking the ecological consequences of nanoparticles.⁷² Additionally, HTS might make it possible to create models that forecast how nanoparticles will behave in biological systems. HTS's objective is to support the safe development of nanomaterials by using quick, automated screening techniques to produce comprehensive, comparable toxicity data for thousands of distinct nanomaterials.⁷³

Computational Models Computational methods made possible by the development of AI and ML allow nanomedicine to investigate safety issues effectively and economically. The safety assessment at the early phases of drug research, which integrates data at several levels and produces reliable conclusions, has a detrimental impact on the medication's failure in the drug development process. Understanding the limitations, science and opportunities that underpin this application is crucial to getting the most out of it. Additionally, computational approaches consider the pharmacokinetic elements in addition to the ligand-receptor docking concept in order to illustrate the results.⁷⁴

Quantitative Structure-Activity Relationship (QSAR) Modeling

Routine nanotoxicity investigations

use less time, money, and resources thanks to computational technologies like nano-QSAR models QSAR and (at the nanoscale). Pharmacokinetic and pharmacodynamic data are primarily correlated with in vivo application scenarios using these models.⁷⁴ QSAR methods use a compound's physicochemical characteristics to predict its biological activity (⁷⁵). Later, in 1988, Cramer and associates created a different method known as 3D-QSAR, which takes into account the interactions, activity & spatial structure of molecules.⁷⁵ One-dimensional (1D), two-dimensional (2D), and three-dimensional (3D) techniques are all covered by Nano-QSAR, making it a very universal model⁷⁶. The low-energy conformations that docked into the ADME model were used to build the 3D nano-QSAR. Atoms in a crystal are more likely to overlap their orbital energy and divide if they are close to one another. Conduction band overlap suggests that NMs are cytotoxic or have other disruptive effects.⁷⁶

Stem Cells as a Novel Approach to Predict Toxicity

All of the biological cell can be developed from pluripotent stem cells. The majority of the precise NMs toxicity is still unknown because of the use of imprecise, oversimplified techniques, such as 2D cell lines, in compound testing and validation. Recent stem cell research has focused more on ESCs and iPSCs.⁷⁷ ESC is produced from an embryo's undifferentiated inner mass cells, whereas iPSCs are derived from somatic cells that have been reprogrammed to function as ESCs by activating genes or forcing the expression of reprogramming genes such Klf4, Oct4 and Sox2.⁷⁸

Omics Technologies

"As a whole" is what the suffix "omics" stands for, and it encompasses proteomics, metabolomics, transcriptomics, epigenomics, and genomics. Adaptive responses can be identified using these techniques. Systems toxicology encompasses transcriptomics, proteomics, metabolomics, genomics, and epigenomics (miRNomics and DNA alterations). Using recombinant DNA, DNA sequencing, and bioinformatics to examine the structure and function of the genome, genomics studies genes and their roles. These technologies' main benefits include the ability to forecast toxicity at low exposure levels to nanoparticles, which can stress cells without causing toxicity.⁷⁹

NPs cause this pattern and affect a number of biological functions, including membrane integrity, inflammation, apoptosis, and proliferation.⁸⁰

CONCLUSION

Nanotoxicology is a rapidly expanding field focused on assessing the risks of nanomaterials used in industries like healthcare and consumer products. While nanotechnology offers significant therapeutic potential, such as in drug delivery and cancer treatment, the unique properties of nanoparticles—such as size, surface area, shape, and charge—can lead to unexpected toxicological effects. Understanding the mechanisms of nanoparticle toxicity, including genotoxicity, inflammation, and oxidative stress, is critical for ensuring their safe use. The biodistribution, clearance, and exposure routes of nanoparticles (inhalation, dermal contact, and ingestion) significantly affect their health impacts. Despite progress, many gaps remain in understanding nanomaterial safety, and more research is needed to establish comprehensive safety standards. Advancements in novel testing methods, including high-throughput screening, computational models, and stem cell-based assays, are essential to assess the long-term effects of nanoparticles and guide their safe development. Both *in vitro* and *in vivo* methods are crucial for evaluating the biological effects of nanoparticles, with assays focused on cytotoxicity, oxidative stress, genotoxicity, and apoptosis being commonly used. Emerging technologies like high-throughput screening, computational models, and omics technologies offer efficient, cost-effective ways to predict nanoparticle behaviour and toxicity. These tools enhance our ability to assess the molecular impacts of nanomaterials, identify adaptive responses, and predict low-exposure toxicity. As nanomaterials continue to evolve, integrating these advanced methods is vital for ensuring the safe development and regulation of nanomaterials, protecting both human health and the environment.

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Clinical Trial Registration

This research does not involve any clinical trials.

Permission to reproduce material from other sources

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Galanki Vasantha : Conceptualization, Supervision, Methodology, Writing- Review & Editing; Rangala Mohini: Writing – Original Draft, Data Collection, Analysis; Iragavarapu Tejolahari: Analysis and Data Collection, Gajula Niharika: Data Collection and Analysis; Allampalli Likhita: Data Collection and Analysis.

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