Chapter

Toxicity Evaluation and Biocompatibility of Nanostructured Biomaterials

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Abstract

Biomaterials have occupied a prominent place in regenerative procedures to restore human health. Moreover, there is a greater need in understanding, analyzing and establishing their toxicity profile. These, when made into nano-sized constructions called nanostructured biomaterials, their regenerative potential is enhanced, which could influence their toxicity nature. This chapter intends to give comprehensive information on their nanotoxicology pathways at the cellular level, their entry pathways into the human body, and their potential consequences on human health. It clearly explains the cytocompatibility and biocompatibility of various nanostructured biomaterials for potential human health applications like drug delivery and tissue engineering. A detailed overview of various in vitro and in vivo evaluation methods of biocompatibility of nanomaterials are outlined in this chapter that researchers should address as they move forward in developing new systems for the field of regeneration.

Keywords: nanobiomaterials, nanotoxicity, cytotoxicity, biocompatibility, regeneration

1. Introduction

Organ and tissue transplantation has limitations like immune reactions and donor limited availability, leading to the necessity for bioactive tissue engineering biomaterials, which is a rapidly developing multidisciplinary field. Nanoparticles (NP) based biomaterials offer better control over their desirable properties, such as the controlled release of biological molecules at surgical sites and optimization of mechanical properties matching recipient sites [1, 2]. Nanotechnology includes the development of NP and their applications in wide areas of interest. The size of NP will be within the range of ~10–1000 nm which can be synthesized in colloid and solid states [3]. Due to the increased production and use of nanoparticles in various fields, the unintended toxicity of nano biomaterials is a growing concern in tissue engineering and regenerative medicine. Minimal data are available regarding their toxicity and end products in the

human physiologic system. Exposure pathways for NP include dermal penetration, ingestion, and inhalation. Physical properties of NP that influence toxicity include surface chemistry, particle shape, and size [4].

This chapter presents toxicological profiles of NP used as biomaterials highlighting in vitro and in vivo analysis techniques. This will give an insight into overall patterns and further requirements for assessing the toxicity of these biomaterials.

2. Human exposure routes to nanomaterials

Nanomaterials or nano-sized particles can enter the body through various routes such as skin, olfactory tracts, weak or damaged skin, intestinal tracts, respiratory tract, and intravenous or intramuscular routes. Their entry can have adverse biological effects such as tumors, cardiac diseases, skin problems, allergic reactions, and respiratory diseases. As a result of human industrial activities, dust storms or volcanic eruptions, humans get exposed to nanoparticles continuously and their toxicity depends on the chemical composition of the nanoparticles. Based on the composition, nanoparticles are divided into inorganic, metallic and metal oxide, where their biological effects and toxicity level are explained. The body parts easily exposed to nanoparticles are skin, gastrointestinal tract and lungs.

2.1 Skin as the entry route

Human skin which has an average surface area of approximately 2 m^2 has a thick outer layer of keratinized dead cells and which does not absorb any essential elements other than solar radiation. Skin appendages and stratum corneum are the major routes of entry through the skin. The root of the hair or follicles gives space for nanoparticles to accumulate; during the follicle opening, they enter into deep layers of skin. Intercellular spaces along the lipid layers are the easiest route for the nanoparticles to penetrate the skin. The classical diffusion theory can understand the diffusion of aggregated particles through stratum corneum. Even though the stratum corneum is porous and allows facile entry of nanoparticles, the dose and exposure time affects the entry. Follicular penetration varies at different sites on the body. It is observed that the follicle morphology on the lateral forehead is the area of maximum penetration as it has maximum surface coverage. Follicle area is considered the best site for particle storage as it is not exposed to washing or fabric contact. This area is considered suitable for drug release and a shortcut to the systemic circulation [5, 6].

Polymeric nanoparticles or drug carriers cannot penetrate the skin without mechanical stress. Several studies have been conducted to study the drug release from polymeric particles through the skin. It has been found that the particles did not penetrate the skin without any mechanical stress. Studies on hairless animals showed that the drug could not penetrate and thus proved that hair follicles pave the way for entry. Most topical applicants or cosmetics include zinc oxide (ZnO) or titanium dioxide (TiO_2) nanoparticles, which are mostly studied for skin penetration. It is shown that the size of the particles and exposure time effect the penetration. Titanium dioxide nanoparticles (TiO_2 NP) of size 20 nm showed their presence in the first 3–5 layers of corneocytes. Very small sized TiO_2 NP 4 nm size showed presence in the deep epidermis layer of pig's ear after 30 days of exposure. ZnO nanomaterials of size 30–40 nm could not penetrate deep into epidermis layer. Liquid type liposomes can penetrate epidermis layer, whereas gel type cannot. Metallic nanoparticles show

higher penetration rates. Another important aspect is the physical state of the skin. The damaged skin easily allows the nanoparticles whereas intact skin only paves the way for tiny nanoparticles below 10 nm to penetrate the epidermis layer [7, 8].

The shape of the nanoparticles also affects toxicity. Differently shaped silver nanoparticles penetrated differently into the various layers of skin. Tak et al. [9] showed that the intercellular penetration pathway plays a central role in the shape-dependent penetration of AgNPs through the lipid matrix between corneocytes. The possible skin penetration pathways are shown in **Figure 1**.

The rate and depth of penetration of nanoparticles affect the toxicity level. Metallic nanoparticles can enter systemic circulation easily and thus can add to the toxicity depending on their dose. Thus, safety evaluation must be done before using nanoparticles in cosmetic formulations and drug delivery. Rapid penetrating nanoparticles pose more toxicity than slow penetrating ones as the latter can have efficient drug delivery and will be attacked by the immunological system of the body. In contrast, rapid penetrating NP can circumvent the macrophages and cause systemic toxicity. Argyria is a diseased condition due to the toxicity of silver nanoparticles leading to permanent pigmentation of eyes and skin [9].

2.2 Inhalation as the entry route

Airborne nanoparticles enter the lungs and cause respiratory disease by accumulating in the alveolar cells. They not only affect the lungs but also affect the

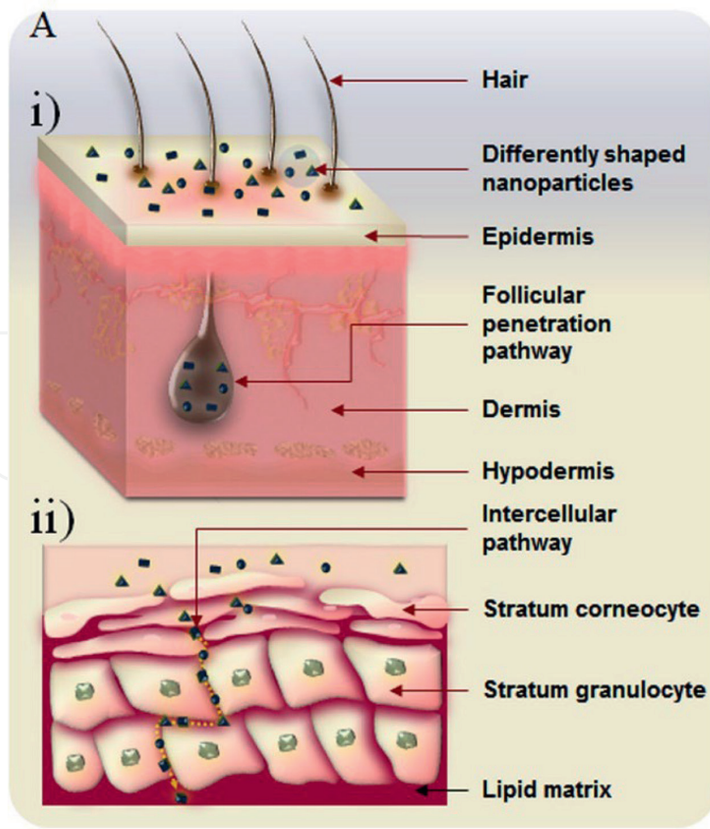

Figure 1.
The schematic diagram of possible skin penetration pathways of three differently shaped AgNPs. (A) Two main possible skin penetration pathways are illustrated: (i) enters via hair follicles (the follicular penetration pathway); and (ii) diffuses through the gaps between corneocytes (the intercellular penetration pathway). Reproduced with Creative Commons Attribution 4.0 International License (CC BY. 4.0) from Ref. [9].

extrapulmonary organs like the heart and liver. Metallic oxide and hydroxide NP cause pulmonary inflammation. The nanoparticles enter through olfactory tubes, pass the pharynx, and reach the alveoli. They have a good retention time, and the diameter of the particles affects the toxicity level. Some NP travel through the alveolar epithelium and capillary endothelial cells and reach the cardiovascular system and other internal organs [10]. Chronic obstructive pulmonary disease (COPD) is one of the major cause of death in the world as suggested by World Health Organization (WHO). Sarcoidosis and pulmonary fibrosis are other respiratory diseases caused by airborne pollutants. Wildfire, dust winds and volcanoes are sources of natural nanoparticles whereas engineered nanoparticles are found in gasoline exhausts, cosmetics and drug delivery carriers. These nanoparticles cause direct injury to lung tissues and cause inflammation. These nanoparticles then move to systemic circulation from the alveolar airspaces and thus cause toxicity to other internal organs. The high surface area of nanoparticles help them interact with enzymes or proteins of the immune system and thus causes inflammation and subsequent injury to the tissues, thus being detrimental to tissues [11].

The level of injury caused by nanoparticles is different in different regions of the lungs based on the clearance mechanism and cell types. In lung alveoli, there is only a single layer of cells; in bronchi and bronchioles, there is a layer of mucus for protection. Thus the nanoparticles which reach the alveoli can easily diffuse into blood capillaries and enter the systemic circulation, causing toxicity. Alveolar epithelial cells (AEC) are critical in protecting the respiratory tract and AEC in alveoli gets easily damaged by NP. AEC I gets damaged easily by NP and to replace it to maintain the alveolar structure, AEC II undergoes hyperplastic proliferation and repeated exposure to NP results in its aggregation in systemic circulation and thus leads to cardiovascular diseases. As cited above, the shape of nanoparticles determines the level of toxicity.

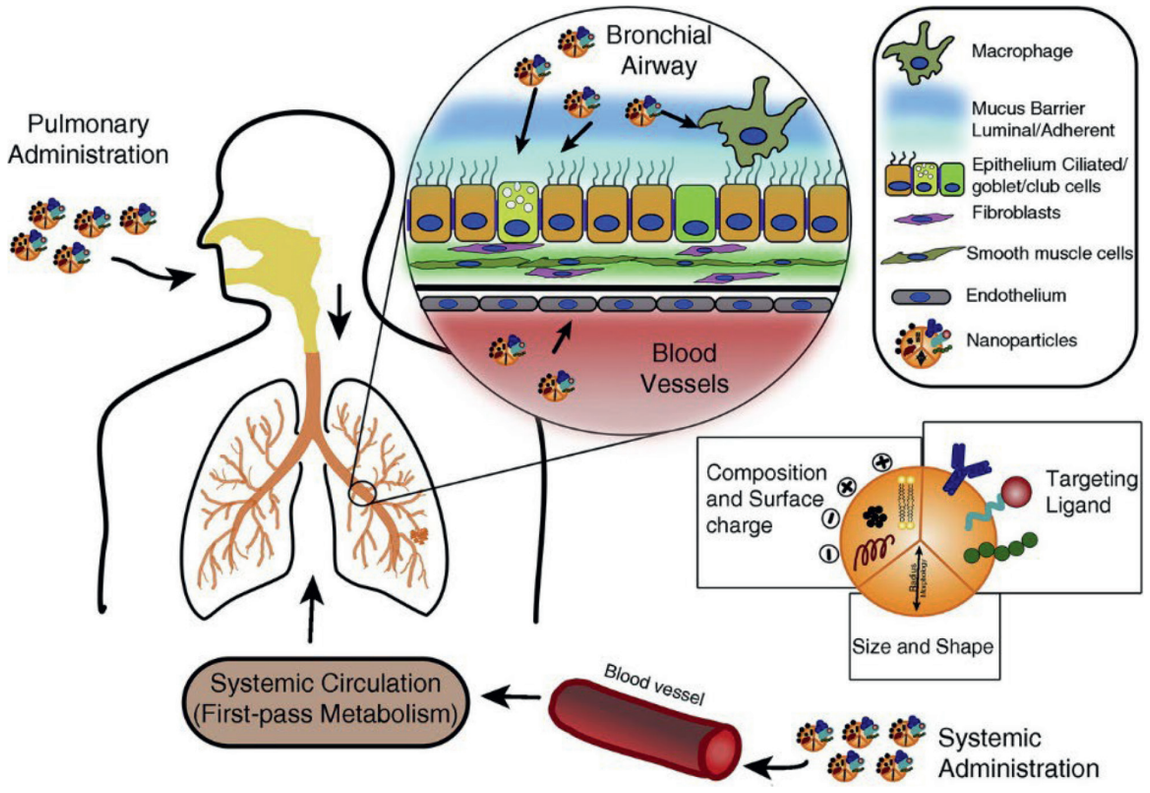

Figure 2.
Systemic and pulmonary administrations of nanoparticles to target respiratory cell types. Reproduced with Creative Commons Attribution 4.0 International License from Ref. [13].

Needle-like and rod-shaped NP cause more cellular destruction than spherical and flake-like structures. Other crucial factors to be considered are NP's degradation, solubility and chemical composition. The more they persist in the system, the more toxic they are to the cells. Metal NP like gold and silver persist in lungs for more than 7 days. This causes toxicity to all organs by creating oxidative stress, thus leading to cellular toxicity [12, 13] (**Figure 2**).

2.3 Nanoparticle ingestion

Metallic NP, carbon-based NP, ceramic NP, polymeric NP or dendrimers are the classes of nanoparticles that enter the gastrointestinal tract through ingestion. Implant materials have become very common, and orthopedic and dental implants use nanoparticle coatings to enhance their performance and bioactivity. However, intentionally or unintentionally, these implants release ions and NP over time, which cause adverse effects in the intestine and its related organs. The gastrointestinal tract (GIT) has a huge surface area of approximately 200 m^2, which is very much amenable to NP interaction. NP are absorbed by the GIT and thus enter the systemic circulation. Nanoparticles also damage the microbiome of the gut and thus affects digestion [14].

Targeted drug delivery through nanocarriers has been used in treating skeletal infections. But in many cases, these engineered nanomaterials create cytotoxicity. In order to achieve native bone tissue structure and more biocompatibility, nanomaterials have been used in orthopedic implants. Dental implants use a wide range of engineered materials, including nanomaterials. Titanium is used as dental implants, which can release titanium NP due to many activities like wear and tear caused by chewing or bacterial activity and chemical or physical deterioration. Titanium oxide nanoparticles from dental implants can dissolve in saliva reaching the intestine and other organs like the liver, spleen, kidney, and heart. Risk assessment of the engineered nanomaterials should be very focused on criteria like physicochemical characterization and thorough biological evaluation as per the regulatory agencies' guidelines [15]. Most implant failures occur due to wear and the release of particles which cause a chronic inflammatory response. A cobalt-chromium hip implant faced rejection due to the release of cobalt and chromium nanoparticles triggering an inflammatory response. A study conducted by Posada, 2015 showed the involvement of lymphocytes in such implant allergies, and within 48 hours of treatment, the metal nanoparticles caused apoptosis in the cells [16].

The most common nanoparticles that enter GIT are silver, iron oxide, titanium dioxide, zinc oxide etc. Organic NP, liposomes, engineered protein NP etc. pose little concern about their toxicity as many of them have been used by humans for centuries. Not many studies have been done in this area of nanotoxicology, and very little information is known about the toxicity of such nanoparticles. But the most important fact to be noted is that the properties of each type of nanoparticle differs on many factors and that these factors determine their toxicity. Dimension, morphology, composition, surface charge, aggregation state affects the fate of the nanoparticles in vivo. Most of the nanoparticles, after passing through various parts of the GIT change their properties due to interaction between the proteins and varying pH conditions. Thus, their fate is altered when compared with that in vitro. The properties of pristine nanoparticles will be very much different from the nanoparticles in vivo and thus, the studies related to their toxicity should be taken into consideration only for in vivo results. Most of the studies which show cytotoxicity in cell lines do not show any toxic effects when performed in animal models unless it is at a very high dose. Thus the in vivo studies should be concentrated more on the physiological aspects of the cells rather than on the organs as a whole [17, 18].

3. Pathways of cellular uptake

In drug delivery applications, the nanocarriers should go inside the cells to efficiently deliver drugs to target organelles or cells. Thus, the uptake route or mechanism of NP into cell should be considered in designing a nanocarrier system. Cell membrane is the protective coating of a cell and is not permeable to all particles as it maintains homeostasis. The lipid molecules of the cell membrane have hydrophilic heads and hydrophobic tails. Lot of small molecules enter the cell by passive diffusion. The cell uptake of molecules with the help of energy from ATP (Adenosine triphosphate) is called active diffusion [19].

There are a lot of factors which influence the internalization of the NP and their interaction with intracellular components such as size, surface modifications, net charge, hydrodynamic volume and stiffness. The persistence of nanoparticles in the cell and their release of free radicals to induce oxidative stress bring toxicity and cell death. Endocytosis, an active transport, is the most important pathway by which molecules enter the cell and trigger responses. The main types of endocytosis are phagocytosis (cell eating) and pinocytosis. In the process of endocytosis, the particle

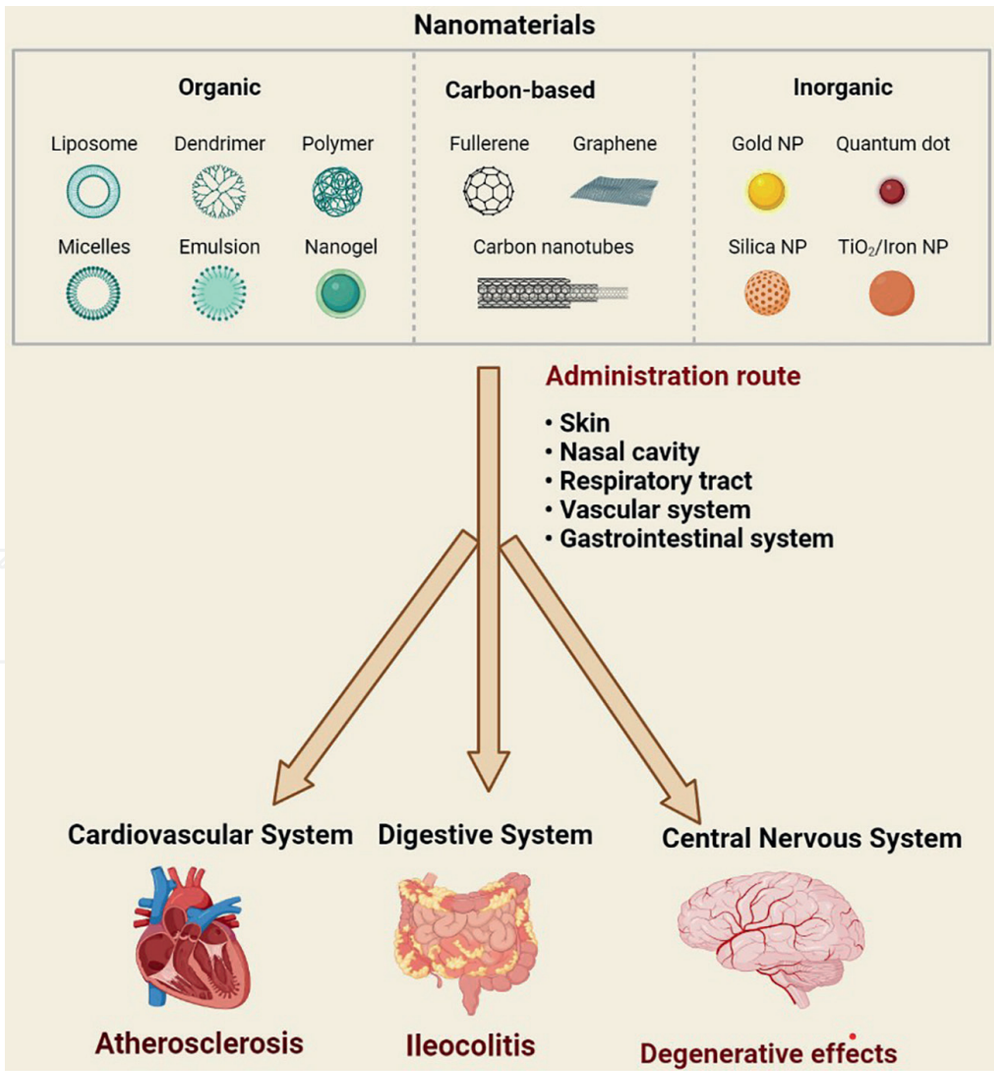

Figure 3.
Schematic diagram showing the types of nanomaterials and their impact on various organ systems. Created with the help of Biorender.com [18].

to be internalized with be covered by a part of cell membrane which the buds off inside the cell to form vesicle containing the particle [20].

Phagocytes are immune cells that protect from infections and pollution by engulfing foreign bodies. The 3 main phagocytes are macrophages, neutrophils and monocytes. In phagocytosis opsonins (group of proteins found in serum) play a major role by adsorbing onto NP surface and form protein corona. Phagocytes recognize these and a signaling cascade is triggered which internalizes the particles forming phagosome [18] (**Figures 3** and **4**).

Different cell types use different uptake mechanisms for the same NP. It can occur through phagocytosis, macropinocytosis, clathrin and caveolin mediated endocytosis, non-clathrin and non-caveolin mediated endocytosis are some among them. Nanoparticles less than 100 nm in size can enter the cells and size less than 40 nm can enter the nucleus. The surface charge of the NP are a crucial factor for cell internalization where the negatively charged cell membrane is attracted towards positively charged NP. Small NPs are engulfed by F-actin mechanism, dynamin and lipid rafts which are energy-dependent. NP of size more than 500 nm are engulfed by phagocytosis and macropinocytosis. Aggregated NPs, TiO_2 or carbon black undergo opsonization in the biological fluid and undergo phagocytosis or macropinocytosis [19].

3.1 Adverse effects of the cellular uptake

The bio resistance of certain nanomaterials to degradation is the root cause of toxicity. These nanoparticles retain in the endosomal compartment of cell. Certain other nanoparticles like ZnO, when taken into the acidic part of lysosome will get dissolved into Zn^{2+} ions which in excess amounts will cause cytokine production leading to cytotoxicity. Non-soluble nanoparticles like TiO_2 and carbon black NP were found to be free in cytoplasm and even in the D.N.A. The reasons for this toxicity could be that these NP diffuse through the cell membrane through transient holes or they may

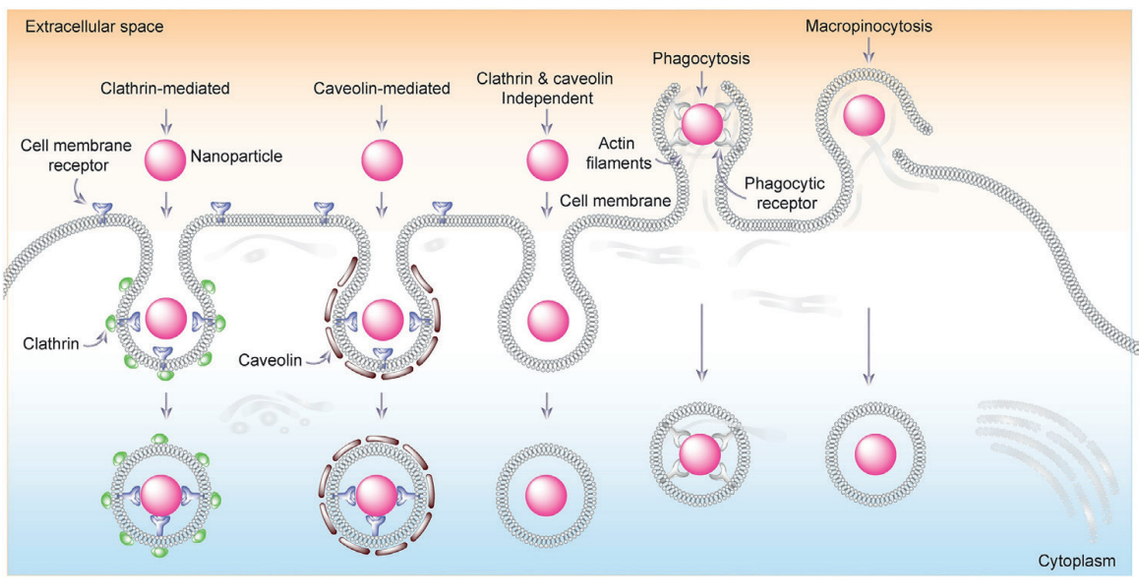

Figure 4.
Schematic representation showing the mechanisms of nanoparticle cellular internalization such as clathrin-mediated; caveolin-mediated; clathrin- and caveolin-independent; phagocytosis and macropinocytosis pathways. Reproduced with creative commons attribution 4.0 (CC-BY-4.0) license from Augustine et al. [19].

be accumulated in the lysosome where later on lead to membrane rupture and release in the cytoplasm. These cytoplasmic NP during mitosis can enter the nucleus through microtubules and are found in the DNA [21].

The engineered NP persisting in the cell result in oxidative stress leading to apoptosis and inflammation. Oxidative stress is a result of imbalance between reactive oxygen species [ROS] and antioxidant capacity of cell. NP trigger the ROS production and thus leads to the imbalance and oxidative stress. ROS has various cellular roles by acting as secondary messengers. If the stress caused by ROS is at a higher level, it can result in cell membrane and organelle injury leading to necrosis or apoptosis. The smaller the size of the nanoparticles, the higher the surface area and thus produce more ROS. Thus, the oxidative stress caused by the small NP affect lipid membrane and cause their disorganization in structure and function. Inactivation of certain sensitive proteins which have methionine or cysteine in their active site are also the toxic effects of the NP which entered the cells. Genotoxicity is also a serious effect of NP which damages DNA because of intrachain adducts and strand breakage [22].

4. Effect of physicochemical properties of nanomaterials

The cellular uptake and dispersion of nanomaterials depend mainly on the physicochemical properties of the particles. Evaluating the role of physicochemical properties in toxicity issues is very important (**Figure 5**).

4.1 Size

The size of the nanoparticle is proportional to its surface area. The lesser the size, more is the surface area and more its reactivity. The size of the particle and its effect in vivo is difficult to observe as its structure changes in vivo. The ability of the nanoparticles to enter the cells and modify the macromolecules accounts for its level of toxicity. The nanomaterials below 100 nm can enter cells easily as their size is comparable with that of protein globules, DNA., and cell membrane thickness. Very small NP of size less than 20 nm which can enter the nucleus are more toxic than particles

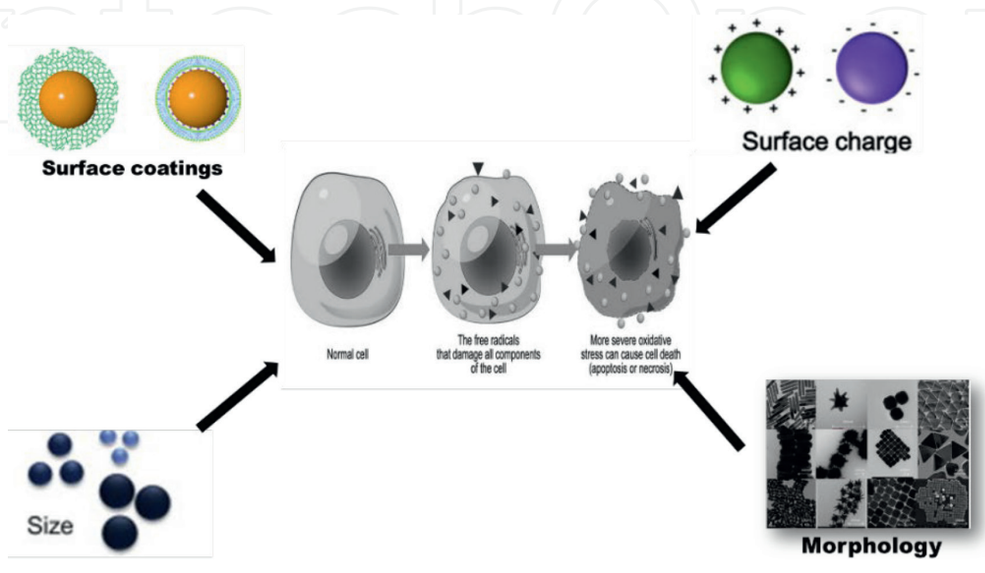

Figure 5.
Schematic diagram showing the various physicochemical properties of nanoparticles and their effect on cell [23].

which cannot enter the nucleus. Studies have done on gold nanoparticles in relation to their size and toxicity in vivo. It was found that particles of size 1.4 nm whose size is comparable with that of a major groove of DNA will easily block transcription by interacting with the sugar-phosphate in DNA. It is shown by Zhang et al. that those particles of size less than 5 nm enter cells through translocation while those above 5 nm enter through specific pathways like macropinocytosis and phagocytosis [24]. Small sized particles circulate rapidly all around the body while particle above 50 nm were found in organs like liver and spleen. Star shaped nanoparticles have shown accumulation in lungs while larger nanoparticles are recognized by the immune system and eliminated from the body. Size and shape influence the kinetics of excretion and accumulation of the particles in vivo. The larger surface area of the particles help in adsorption onto the cell surface where particles above 600 nm destructed the cell membrane and caused haemolysis. In contrast, particles of size 100 nm did not cause membrane destruction [23–25].

Larger particles show negligible toxicity when compared with smaller particles. The shape of the particles also makes them behave differently. Spherical particles are easier to be engulfed by cells and they are the ones that cause less toxicity. Whereas rod shaped and fiber-shaped nanomaterials are difficult to eliminate and their toxicity is higher when compared to that of their spherical counterparts. Nanoparticles that enter the body can agglomerate or agglomerates can disperse into primary particles. When primary particles come in contact with lung fluid, they agglomerate. Agglomerates or particles of size >1 μm are up taken by phagocytosis and their clearance mechanism is easy. But the smaller the particles or the agglomerates, the more difficult it becomes to eliminate them and toxicity like genotoxicity happens. The more time they retain in the body organs like lungs, the chances of depositing in secondary organs is more and they damage the lungs. Clearance rate by translocation is less than 0.5% compared to their exposure and these can result in their persistence, causing toxicity and organ damage [26].

4.2 Morphology

The shape of particles is very important in determining their fate inside the body. In nature we can see different shapes for microorganisms like spherical shaped HIV virus, star shaped bacteria and rod shaped TMV which confer different effects in vivo. Thus the function of a nanoparticle with a specific shape determines their effect in nature. The nanoparticle uptake, toxicity, biodistribution and inflammatory response is highly dependent on the structure of the nanoparticle. It is studied that rod shaped particles enter inside the cell easily even compared to spherical particles. But the shape alone does not add to this effect, we need to consider the aspect ratio where higher aspect ratio particles expose more to the cell surface thus easily engulfed into the cells. Nanowires and nano worms due to their length have higher aspect ratio and they enter cells with ease. Particles with highly curved and sharp edges are not very easily up taken by cells. High aspect ratio particles have shown proinflammatory effects leading to cytotoxicity when conducted studies in in vitro models. The persistence of nanoparticles in vivo depends on the geometry where it has been found that filament-shaped micelles prolong the persistent time compared to spherical-shaped micelles. In a study conducted on gold nanoparticles in ovarian cancer xenograft by Arnida et al., it was shown that rod-shaped gold particles were more in circulation and accumulated in tumor cells than spherical-shaped gold NP [27].

The behavior of nanoparticles in vivo cannot be related to just one physicochemical factor alone, whereas the toxicity or fate of a nanoparticle depends on many factors like size, shape, ligands, material, charge, route of exposure etc. Among these factors, the surface chemistry of a nanoparticle has more influence than the particle's geometry since receptor ligand binding strength is the limiting factor which determines the uptake and thus leads to toxicity of these NP. But when you consider the same surface chemistry of a rod shaped and sphere-shaped NP., the rod-shaped NP will be favored by the cells. Apart from this, the fluid motion of rod-shaped particles is favorable. Spheres and short rod NP accumulate in liver, higher aspect ratio NP are found in spleen and lungs. Nanofibers that enter through lungs have more cytotoxic effects where the asbestos particles inhalation is an example. They can disrupt the cell membrane's lipid bilayer and cause a pro-inflammatory response [28].

Nanoparticle toxicity strongly depends on their morphology. In studies conducted on gold nanostructures, it was found that gold nanostars bind to serum proteins better than gold rods. Spherical gold nanoparticles showed higher binding affinity when compared with branched particles. The radius of curvature of the nanoparticles is a factor for protein corona formation. A planar surface is provided by a large radius of curvature, thus resulting in more effective protein binding [29].

4.3 Surface charge

The cytotoxic effects of nanoparticles depend on their charge density and charge polarity. Positively charged nanoparticles of gold, zinc oxide and silica have more profound effects than their counter parts. For certain nanoparticles of polymers, the charge does not have much effect on their properties in vivo. Some nanoparticles which are porous does not depend on charge such as mesoporous silica nanoparticles. But phagocytic cells interact more with negatively charged particles. In case of nonphagocytic cells where they interact with cationic charges on NP surface, its cellular uptake is more and thus cause cytotoxicity by plasma membrane disruption. But the effect of serum reduces their uptake. Whereas phagocytic cells interact more with anionic NP and ingest them effectively and serum has a positive effect on their uptake [30].

Cell type is an important factor which determines the uptake of NP. The same NP behave differently in various cell types. Effect of surface charge on Mesoporous silica NP in human mesenchymal stem cells revealed that a strong positive charge on the NP showed a good uptake in mesenchymal stem cells whereas their uptake was inhibited in 3 T3 L1 cell lines. When the positive charge density is less, the uptake efficiency is low [31].

Ionic Interactions of NP and cell membrane is important in understanding the fate and effect of NP in the living system. Even though the cell membrane is negatively charged, it behaves differently to positively charged molecules depending on the charge density and other factors. Gold nanoparticles of different surface charges (positive, negative, neutral, zwitterionic) were applied to various cell types. It was shown that positively charged AuNPs depolarized the membrane more than their counterparts. The authors also suggested that changing or varying the surface charge density or applying both positive and negative charges on the nanoparticle may result in an organized cellular uptake and thus target various organelles [32].

The toxicity of silver nanoparticles in the environment and to biological systems is greatly influenced by the surface charge of silver nanoparticles. The electrostatic barrier between silver NP and cell membrane highly affects the fate of NP and influence of other factors such as shape and size become negligible. The authors suggest

that surface charge measurement of NP can be used as an analyzing tool to find the toxicity of the NP [33].

Curcumin which is considered as a boon for many diseases can become toxic to cells, especially alveolar macrophages when the surface charge is altered. Curcumin nanoparticles when given positive charge by coating with the polymer polyvinylpyrrolidone and was given negative charge by coating with polyvinyl alcohol and neutral with dextran. The surface charge varied from −20 mV to +5.5 mV. The positively charged curcumin NP resulted in lysosomal and mitochondrial destabilization leading to ROS generation and apoptosis. They entered the cells through clathrin-coated endocytosis and damaged the lysosomes. Thus the effect of surface charge on materials, especially nanomaterials are a very important aspect to be looked upon to determine cytotoxicity and during the designing of nanoparticles [34]. Apart from these facts, another important factor is the density of cationic charges. When the density of the cationic charge is less, the toxicity effect will also be less. The zeta potential of the Nanoparticles does not have any effect if the surface charge density is very high [35].

4.4 Coating

Nanoparticles are given coating with various materials depending on the result to be achieved. When they are used for cell targeting, the NP must be coated with protein ligands identified by the cell receptors or if targeted to cancer cells then attached with RGD sequence. These coatings can affect the properties of nanoparticles when used in pristine form. The coating materials determine the toxicity of the NP, where they can increase or decrease the toxicity levels. There are a plethora of coating mechanisms and some methods prefer coating the NP with more than one material. Silver nanoparticles tend to aggregate, thus, coating helps improve the NP's stability in suspension. Certain coatings make the silver NP more toxic such as citrate compared with PVP, or protein coated when compared to bare silver NP [36].

Type of coating material, whether they are hydrophilic or hydrophobic, influences the toxicity of metal-based nanoparticles. Hydrophobic coatings have proved to be safer than hydrophilic coatings and this also depends on the density of the coating. Zinc oxide used in various cosmetic formulations when coated with hydrophobic material showed very little toxicity and is recommended for commercial use. This can be explained by the reduced bioactivity of the hydrophobic particles. Theoretical models such as QASR quantitative structure-activity relationships should be applied to evaluate the effect of various coating on nanoparticles [37].

5. Physiological impact due to nanomaterials

Nanostructured biomaterials are being analyzed in detail currently for regenerative applications. However, their physiological impact due to the prolonged presence of these foreign agents within the body or their degradation byproducts can be broadly divided as the impact of ROS generation and oxidative stress, inflammation and cellular injury due to nanoparticle dissolution.

5.1 Reactive oxygen species (ROS) generation and oxidative stress

Nanoparticles, including transitional metals, have been reported to be oxidized and release reactive oxygen species of which hydroxyl radicals are considered to cause

maximum damage, including affecting cell signaling pathways and affecting the cellular lipids, protein production, alteration of DNA and gene transcription [38]. Also inflammatory cells including neutrophils and macrophages are attracted by phagocytosis of these nanoparticles which in turn leads to the production of proinflammatory cytokines and oxidative stress [38]. Researchers have reported the extent and severity of inflammation is dependent on characteristics of the nanoparticles such as size and shape.

In response to low levels of oxidative stress generated by nanoparticles, the cells release antioxidants such as ferritin, N-acetylcysteine (NAC), which nullify the oxidative stress; however cellular damage occurs due to excessive reactive species [38].

5.2 Inflammation

Deng et al. reported that the decreased size of poly aryl acid-coated gold nanomaterials (<20 nm) have been associated with the activation of the Mac-1 receptor of the monocytes [39]. This in turn, leads to upregulation of the proinflammatory cytokines via the NF-κB pathway. Poly(d,L-lactide-co-glycolic acid) (PLGA) nanoparticles 75 nm or lesser reported lesser PMN in bronchial lavage fluid than in 200 nm size particles. Also, the varying shapes of these nanoparticles have been found to elicit inflammatory responses. Albanese et al. reported that the size and shape of these nanoparticles dictate the ligand and receptor interactions that in turn determine the cellular uptake and the further downstream reactions [40] However, nanoparticles can be used as drug-delivery agents to suppress these inflammatory reactions.

Nanoporous scaffolds similar in architecture to the native tissues have been found to have lesser associated inflammatory reactions [41]. Silica and hydroxyapatite based nanoporous scaffolds have integrated better than cancellous bone substitutes in association with implant-based in vivo studies. Nanoporous scaffolds made of native matrix proteins or with a coating of bioinert polymers (such as poly ethyl glycol PEG) along with sustained release of anti-inflammatory agents and antioxidants would have better integration with host tissues with minimal inflammation.

Certain nanotopographical alterations in these nanomaterials are capable of altering the host inflammatory response attenuating or at other times exacerbating the same [41]. It needs to be understood to ensure successful incorporation of nanomaterials in tissue engineering applications.

5.3 Cellular injury due to nanoparticle dissolution

Nanoparticle dissolution is a crucial property which determines the extent of its availability, its toxicity and also its impact on the host environment. Their greater surface area leads to increased physical and chemical interactions which leads to dissolution.

It may lead to cell death or in case of non-degradable NP can accumulate within the cells leading to damage. Quantum dots localize in varying cellular locations, Silica (40–80 nm) is deposited in the nucleoplasm, and gold nanoparticles have been found in the major groove of DNA (leading to the formation of human cancer cells) [38]. Nanomaterials have been found to induce autophagy which occurs as a response to the cellular changes in the aftermath of oxidative stress.

In vitro studies have shown that cationic Polyamidoamine (PAMAM) dendrimers are more likely to lead to autophagy than the anionic dendrimers [41]. Nanomaterials are thought to alter the autophagic degradation activity which might lead to toxicity [42]. Gold nanoparticles as compared to Silicon dioxide nanoparticles have been found to

lower the lysosomal degradation which in turn affects the normal functioning of the cells. Atomic Force Microscopy studies on osteoblast cells reveal alterations in osteoblast cell membranes leading to changes in cell adhesion in response to nanoparticles [43]. Also, Bhabra et al. reported that nanoparticles (Cobalt-chromium) also indirectly affect the cells, including DNA damage without even permeating through the cellular barrier [44].

6. Protein corona formation

When nanomaterials are exposed to biological fluids a dynamic covering of various serum proteins and biomolecules attach to the nanomaterial and this structure is called protein corona (PC). The PC formation on NP can influence its toxicity and targeting behavior due to immunological effects [45].

PC has two different layers one is a hard corona which is the first tightly bound layer and another is soft corona which is the second layer which consists of proteins that are easily exchanged. The composition of corona can change any time by replacement of proteins, but the quantity will remain constant. Initial proteins that bind to the NP will be the most abundant in the blood or the biological fluid exposed to the NP later on the proteins are replaced according to the affinity. This is the 'Vroman Effect' which explains the change in the composition of the protein corona on NP surface in response to time. The proteins compete to get adsorbed onto NP surface. Different types of corona are formed based on the binding affinity and surface ligands on the engineered NP.

Engineered nanoparticles have high free energy and thus adsorb protein to a low-energy state by increasing their dispersion. In less than 0.5 min of exposure to blood plasma, silicon NP adsorbed almost 300 proteins onto its surface [46] (**Figure 6**).

Toxicity of the NP are greatly influenced by the protein corona adsorbed. The fate of an engineered nanoparticle is different in cell lines and in vivo. According to the type of proteins adsorbed, the targeting of the NP and their cellular uptake also changes. There

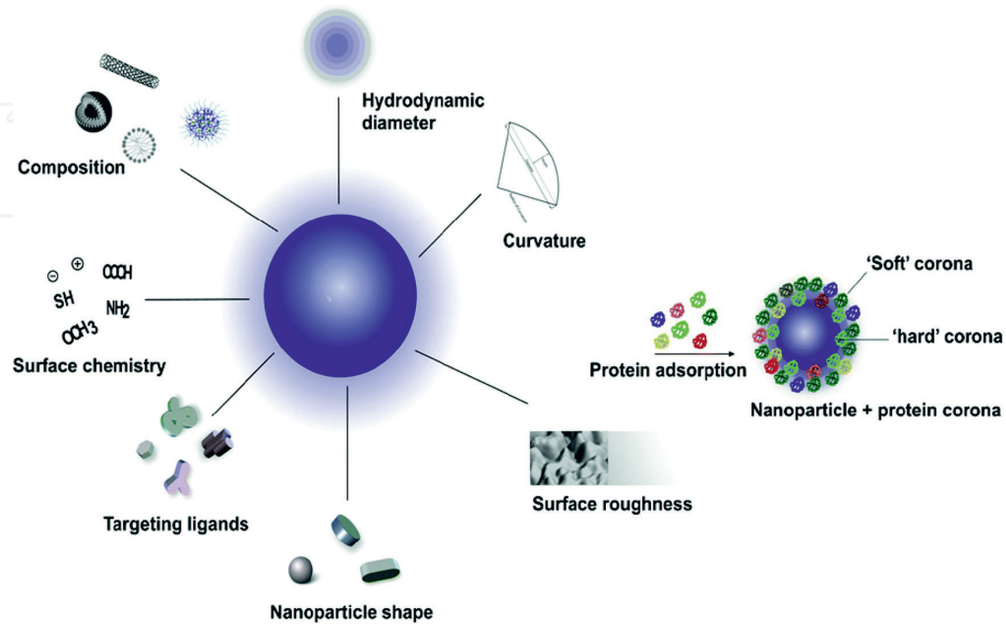

Figure 6.
Factors influencing protein corona formation on nanoparticles. Reproduced with permission from RSC with license id 1257299-1 [47].

are chances of non-specific uptake by receptor-mediated endocytosis. Opsonization plays a major role in determining the fate of ENPs. Proteins act as opsonin and these NP are engulfed by macrophages, it can also lead to uncontrolled aggregation. The protein corona, when made by cell penetrating peptides and antibodies will result in active targeting. It is observed that certain protein corona like blood proteins reduce the toxicity of carbon nanotubes when compared to the fate in cell lines. Overall, the protein corona affects the cellular uptake, target organ and thus toxicity [47].

7. In vitro nano biomaterial cytotoxicity assessment

The minimal ethical concerns associated with in vitro assays make them in demand for toxicity assays. It is much faster, and the cost is also reasonable. It is further subdivided into a few.

7.1 Experimental models used

7.1.1 Cell lines

Various types of cell lines are used in the study of the cytotoxicity of nanoparticles. Using various cell lines for toxicity assay has added risks, which have been documented by Donaldson et al. [48]. This is because of the different toxic responses that the cells experience in vitro when compared to in vivo. Moreover, when carcinoma cell lines are used for in vitro toxicity testing of nanoparticles, the results may conflict with that of normal cells, as the carcinoma cell lines have different pathophysiology than normal cells [49]. The various types of cell lines that are used include murine cell lines (mouse fibroblast cells, mouse macrophage cells, rat mesenchymal cells, etc.), mammalian cells, human osteoblast cell lines, human alveolar and bronchial epithelial cells, human hepatocytes, human macrophages, cancerous cell lines (lung cancer cells, hepatocellular carcinoma cells, colon and cervix carcinoma cell lines, human epidermoid like carcinoma cells—HELA, etc.) and various hematic cells from murine, and mammalian species and humans [50].

7.2 In vitro cytotoxicity assessment methods

7.2.1 Proliferation assay

The cell viability assay is the most crucial investigation to understand a foreign agent's toxicity. Cell viability confirms the number of healthy cells in a sample indicated by their proliferation potential. It also gives information on cell death in the given sample and monitors cytotoxicity [51].

The most prevalent cell viability assays are based on the estimation of the metabolic activity of the cells. Examples are the MTT assay, protease activity assay, and the reduction of resazurin salts [52]. The mitochondrial and the cytoplasmic enzymes in the viable cells can react with these substrates to bring out colored products or fluorescence, which corresponds to the number of live cells.

The most commonly involved method is the MTT assay where a tetrazolium dye MTT (3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide) penetrates the cells and due to the activity of mitochondrial enzymes get converted to formazan, a colored product. They are insoluble crystals and accumulate within the

cells, which are later solubilized using reagents like sodium dodecyl sulfate or isopropanol. The absorbance is then recorded, and maximum absorbance indicates higher survival [53, 54]. With an increasing number of viable cells, the intensity of color deepens. The advantages are quick results and the requirement of limited manipulations [55]. The positively charged MTT dye can readily penetrate the cell membrane. There are negatively charged tetrazolium dyes too, like the MTS (3-(4,5-dimethyl-2-thiazolyl)-5-(3-carboxymethoxyphenyl)-2(4-sulfophenyl)-2H-tetrazolium), XTT, and WST-1(2-(4-iodophenyl)-3-(4-nitrophenyl)-5(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt) that can penetrate the cells only in the presence of an electron acceptor. Since the end products are soluble, they are also preferred.

In protease viability assay, a marker known as glycylphenylalanyl-aminofluorocoumarin (GF-AFC). The aminopeptidase enzyme in the cytoplasm of viable cells acts upon this substrate breaking down the compound into glycine, phenylalanine, and aminoflourocoumarin (AFC) that exhibits fluorescence corresponding to the number of viable cells [56].

Alamar blue/Resazurin dye (7-hydroxy-10-oxidophenoxazin-10-ium3-one) method is more sensitive, offers simpler sample preparation protocols and is inexpensive compared to MTT. The deep blue-colored resazurin gets converted to pink-colored resorufin by the activity of inner mitochondrial enzymes. It is less successful due to difficulty in the biochemical assaying and unwanted reactions [53].

ATP cell assay is also a preferred method for assessing cell viability. The advantages include faster results, higher sensitivity, and lesser artifacts. The principle behind this is that cells with damaged membranes cannot synthesize ATP and cellular ATP concentration gets depleted faster by the endogenous ATPase.

The clonogenic cells assay is a qualitative assessment that investigates the proliferation capacity of cells. The cell sample is exposed to a known quantity of nanomaterials and allowed to proliferate. After 1–3 weeks, they are stained and quantified per growth in number or size. A survival curve is plotted based on percentage survival and increasing dose of nanomaterials. Increasing dosage gradually results in a lower number or size of colonies [57].

DNA synthesis cell proliferation assay is where a radioactive tracker like 3H-thymidine is incubated with a cell sample. This gets incorporated into the DNA of proliferating cells. The radioactivity of the DNA of the daughter cells is measured using a scintillation beta counter, thereby quantifying the number of viable cells. Another method is to use a non-radioactive compound which is 5 Bromo-2′-deoxyuridine (BrdU). BrdU-specific antibodies are then used to obtain a colorimetric estimation [58]. Developed thymidine analogues like 5-ethynyl-2′-deoxyuridine (EdU), 5-iodo-2′-deoxyuridine (IdU) and 5-chloro-2′deoxyuridine (CldU) are also sensitive methods that to have additional advantages over BrdU assay [59].

A precise cell viability assay available is flow cytometry. A cell suspension is introduced into the flow cytometer which allows particles of less than 150 μm to pass through. During the passage, a laser light beam of a known wavelength interrogates the solution, interacts with every single cell, and gets scattered. These scattered lights are converted to voltage signals, the intensity of which gives information on cell viability and cellular kinetics.

7.2.2 Apoptosis assay

Another important parameter observed in nanoparticle toxicity assays is the occurrence of apoptosis. Studies have confirmed that exposure to silver nanoparticles

has resulted in apoptosis and DNA damage with the release of apoptosis markers viz. caspase-3 and caspase-9 [60]. Various tests for assessing apoptosis in the cell culture systems are as follows.

Annexin-V and propidium iodide (PI.) are two cell death markers that give information on apoptosis. The activation of the caspase-dependent pathway during apoptosis causes externalization of the plasma membrane, which is indicated by increased fluorescence. This is because of the binding of Annexin-V to phosphatidylserine. PI is usually impermeable, but it can stain the nucleus when the integrity of the cell membrane is lost. This also relates to the later stages of apoptosis [61].

Comet assay, or the single cell gel electrophoresis assay (SCGE) is a sensitive test can be used both in vitro and in vivo. It detects the breaks in the DNA strands of individual cells [62]. The basic principle is that when an electric current is applied to the cell system, the damaged DNA fragments migrate out of the cell, whereas the undamaged DNA will remain within the nucleus. A comet shape can be seen with undamaged DNA forming the head and damaged DNA forming the tail. The size and shape of the comet give information about the extent of DNA damage. DNA-specific fluorescent dyes will stain the samples, and later the amount of fluorescence in the head and the tail portions, as well as the tail length, are analyzed [63, 64].

The morphological changes specific to apoptosis are another parameter of importance. Irregular reduction in the cell size and fragmentation of DNA confirms the presence of apoptosis. Typical ladder-like patterned DNA fragmentation and irregular cell sizes can be easily identified by Agarose gel electrophoresis [65].

TUNEL assay or the terminal deoxynucleotidyl transferase dUTP nick end labeling assay—it is a method of staining initially described for identifying cells that have undergone programmed cell death and DNA fragmentation [66]. Later it was confirmed that the test could also detect DNA damage due to non-apoptotic events like necrosis [67]. Hence it cannot distinguish between apoptosis and necrosis. It is based on the activity of enzyme terminal deoxynucleotidyl transferase on fluorescent-labeled dUTP. The cells with fragmented DNA will bind to the fluorescent labeled assay molecule and later be estimated by fluorescent microscopy or by immunohistochemical staining. This method gives a quantitative estimation of the viable cells [68].

7.2.3 Necrosis assay

Necrosis assay determines the viability of cells by identifying the loss of membrane integrity. The dye exclusion method is a preliminary method to identify dead or the dying cells. Dying cells exhibit a loss of membrane integrity, so there will be permeation of the dye within the cell. The most used dyes are trypan blue, eosin, or propidium iodide. The cell suspensions are added with the dyes and manual cell counting using a Neubauer hemocytometer conventionally to determine the number of live cells [69].

PI dye can stain the nucleus and binds to the DNA of nonviable cells forming a fluorescent complex. The amount of fluorescent light emitted is proportional to the voltage signal output. The higher the output, the higher the number of nonviable cells will be. Even though the method is faster and more convenient, it is time-consuming and requires expensive instrumentation. A recently designed microchip and microcell counter (Adam, Nanoentek, Seoul, Republic of Korea) is established on which PI stain can be used to distinguish viable and nonviable cells by direct cell counting technique [70].

Neutral Red is another dye that is weakly cationic and slightly acidic in nature. They can easily diffuse through the plasma membrane and get concentrated in the

lysosomes binding to the anionic sites of the lysosomal matrix [71]. Exposure of cells to nanoparticles leads to cell surface alterations and increased lysosomal fragility that favors binding of neutral red thus helping in identifying viable and dead cells [72].

7.2.4 Oxidative stress assay

Exposure to nanoparticles leads to the production of reactive oxygen species (ROS) and reactive nitrogen species (RNS) [73]. These compounds can be detected by X-band electron paramagnetic resonance (EPR) [74], fluorescent probes, and non-fluorescent probes. EPR use is limited due to its high cost. Fluorescent probes are cost-effective but inefficient due to high reactivity, which gives misleading results [75].

Oxidative stress can also be assessed by measuring the lipid peroxidation BODIPY-C_{11} assay. ROS can induce membrane lipid peroxidation. BODIPY-C_{11} is a fluorescent dye that inserts into the lipid bilayers and helps in identifying the oxidized and unoxidized lipids by their respective green and red colors. Later these are quantified fluorimetrically [76].

ROS-induced membrane lipid peroxidation TBA assay for malondialdehyde [77] also gives information on oxidative stress. Various other assays like the measurement of lipid hydroperoxide using Amplex Red assay, antioxidant depletion by 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB), and superoxide dismutase activity by Nitro blue tetrazolium assay is also convenient procedures [78].

7.2.5 Endotoxin assays

Nanoparticles can absorb contaminants onto their surface, resulting in an exaggerated inflammatory response as seen in the case of endotoxins. This acute inflammatory response results in the activating the signaling cascades and releasing inflammatory mediators like cytokines.

The main assays in this regard are the gel clot assay, the coagulogen-based (turbidity) assay, and the chromogenic assay. In the gel clot method, the endotoxin sample solution is combined with LAL solution (Limulus amebocyte lysate) leading to cleavage of coagulogen, which is then checked for subsequent clotting. Chromogenic assay substitute chromogens for coagulogen and releases chromophores when cleaved. Commercial kits like Endosafe®-PTS (Portable Test System) based on chromogenic LAL assays are also available [79, 80].

8. In vivo nano biomaterial cytotoxicity assessment

8.1 Experimental models used

8.1.1 Animal models

Animal models are particularly useful in studying those aspects of nanomaterials that cannot be mimicked by in vitro systems, such as particokinetics (absorption, distribution, metabolism, elimination: ADME), which are caused by cellular responses of the nanomaterials in vivo, and nanomaterial-caused intercellular communication, multicellular interactions, and immune regulation, all of which may be quite difficult to study in vitro [81]. Therefore animal experiments are indispensable in nanotoxicology.

The selection of the animal models is the first and critical step, as a predictive animal model is still underdetermined, although many models have been built. Mice, rats, zebrafish, rabbits, and *Caenorhabditis elegans* are used in nanotoxicology studies, while mice and rats are more appropriate models due to their explicit genome for toxicity tests of organs. Laboratory mice are the most used mammalian research model, popular because of their availability, size, relatively low cost, and ease of handling. Hamsters, as they have wide cheek pouches and mouths that can be widely opened, are used to study pulmonary clearance and toxicity of nanomaterials. Rabbits are also frequently used as they are mildly tempered, easy to confine, and breed. Their ease of handling benefits intravenous injections, blood sample collection, and dermal tests. Zebrafish (*Danio rerio*), a tropical freshwater fish, have a small size, short life cycle, high gene homology with superior animals (also human), and an availability of a wealth of genetic databases, which makes them effective in vivo models to study the toxicity of nanomaterials. Adult zebrafish may be exploited to study nanomaterial transport, biodistribution, bioaccumulation, and chronic toxicity [82].

For the selection of suitable animal models for the assessment of nanomaterial toxicity, Christop et al. [83], suggested the following: the animal model chosen for the toxicity assessment should be suitable in such a way that the pathways of NP penetration into the organism and excretion from the organism conform from the biochemical and anatomical perspective. For the assessment of nanosafety, using a healthy animal may not be suitable when the results are to be extrapolated to the general population, as interorgan compensatory mechanisms may lower the toxic effects of the nanoparticles. Therefore, they suggested that the suitable test animal model choice should be based on the intended application of engineered nanomaterial. Healthy animal models are suitable only for nanoparticle-based therapeutics, and special animal models with unique features should be used when nanoparticles for therapeutic approaches.

8.1.2 Organs

In vivo assessments include analysis of tissue structure changes, apoptosis, and inflammation infiltration in main organs (kidney, spleen, lung, brain, and heart). Target organ systems include those that may concentrate nanoparticles due to their structural specificities, such as hepatic sinusoid and Kupffer cells and the renal filtration membrane [84].

Hepatic assessment: Immunohistochemistry to detect liver fibrosis and inflammation, serum enzymology for hepatic function analysis, hematology and chemistry analyzer for comprehensive analysis.

Renal system: Histopathology and immunohistochemistry, kidney indices for assessment of renal function, and renal variables assessed from blood and urine.

Gastrointestinal system: Histological assay of the GIT, absorption function measured indirectly by the evaluation of metal content and electrolyte.

Pulmonary system: MRI to visualize the pulmonary accumulation of nanoparticles, intratracheal instillation for long-term and short-term toxicity assessments, LDH index, and assessment of oxidative stress.

Cardiovascular system: Assessment for signs and symptoms of phlebitis, hemolysis, thrombosis and signs of cardiac injury.

Nervous system: Assessment of drug delivery of solid lipid nanoparticles across the blood-brain barrier, by using various radiographic techniques such as PET and PET/CT.

Immune and reproductive system: Any modifications of the nanoparticles are assessed to understand their immunogenicity, the generation and release of inflammatory mediators are examined upon the use of nanoparticles, histological changes of major immune organs are assessed by H&E staining, long term studies on the reproductive organs, reproductive index and offspring survival, growth and development are assessed [84].

8.2 In vivo toxicity evaluation methods

In vivo methods of assessment of nanomaterial toxicity have certain advantages over in vitro testing methods. The advantages lie in the fact that in most of the in-vitro testing methods, the growth medium that is used for culturing the cells is supplemented with the lowered concentration of serum proteins as the source of nutrition, and usually, serum of bovine origin is used. The concentration of the serum proteins in the medium influences the nanoparticle-cell interaction, which may range from mild effects on the cells to cell death. A study to assess the role of serum concentration on nanoparticle-cell interactions was conducted by Kim et al. [85]. They suggested that while comparing the results of various in-vitro studies, the serum concentration in the medium used to culture the cell lines should be considered. Moreover, when serum of bovine origin is being used, it may not represent a condition relevant to real in vivo exposure situations.

In vivo toxicity assessment methods assess the toxicity and reactions of the nanomaterials in the organism from the point of administration through the subcutaneous vein, inhalation, skin, oral administration, and intraperitoneal routes; interaction with biological components (such as cells and proteins); spread to different organs, metabolism, penetration into the cells of organs and their excretion [86]. The nanomaterial is introduced into the body of the test animal, and the biodistribution, clearance, hematology, serum chemistry, and histopathology are monitored. This provides more practical data on the interaction of the nanoparticles with the immune system, proteins, and dynamic body fluids at the systemic level.

In order to evaluate the acute *in vivo* toxicity of nanomaterials, the Organization for Economic Cooperation and Development (OECD) guidelines recommend oral toxicity test, eye irritation, corrosion and dermal toxicity, and lethal Dose 50 (LD50).

OECD (Organization for Economic Cooperation and Development) guideline 423 provide data on the methods to be used to determine the LD_{50} (lethal dose 50%) of the nanoparticles being studied. The vehicle used to carry the nanoparticle dose must be non-reactive, and particles must disperse appropriately in it.

8.2.1 Acute oral toxicity assessment

Different concentrations of the nanoparticles may be administered to the test animals orally. Changes in body weight, behaviors, and other toxic signs and symptoms may be recorded regularly. The animals may be observed daily for 14 days for skin symptoms like edema, erythema, ulcers, body scabs, discoloration, and scars. Toxic signs like weight loss, water and food consumption, and the animals' behavior are also assessed. Skin biopsies and blood may be taken periodically for histopathological evaluation and biochemical and hematological investigations, respectively. After 14 days, the animal may be sacrificed, and all organs collected. The following formula may be used for the calculation of organosomatic index: [weight (g) of the organ/total body weight (g)] × 100 [87, 88].

8.2.2 Acute dermal toxicity assessment

Tests may be performed on animals using the test guideline 404 published by the Organization for Economic Co-operation and Development [89, 91]. The substance to be tested may be applied to a small area of the skin of the experimental animal in a single dose for an exposure period of 4 h. The animals will to be examined for signs of erythema edema during the next 14 days. Skin biopsies are taken periodically, and after 14 days, the animal is sacrificed, and the skin is collected for histopathological examination.

8.2.3 Acute eye irritation and corrosion assessment

The standard eye irritation test established by the Organization for Economic Co-operation and Development (OECD) for the testing of chemicals is used as the standard to measure nanomaterials eye toxicity. Five minutes prior to the application of the test substance, 2 drops of a topical ocular anesthetic is to be applied to minimize pain or distress and then the test. One eye may serve as a as a reference control. A small amount of the test substance (0.1 g or 0.1 ml of its colloidal suspension) may be applied to the conjunctival sac of the test eye. The animals are to be observed for toxic symptoms periodically at 1, 24, 48 and 72 h, and grading for ocular lesions of cornea, iris, conjunctivae, and chemosis may be done over 14 days according to OECD test guideline TG 405 [90, 91].

8.2.4 Biodistribution studies

Biodistribution of the nanoparticles may be detected in live or killed animals after conjugating with a radioactive label or organic dye and tracking in blood and tissue at different periods. Some metallic nanoparticles intrinsic properties may be probed by using specific instruments. These tests are especially important for nanomaterials used for drug delivery. Suspension of drug loaded nanoparticles may be radio-labeled and administered intra-venously into the test animal. Blood is collected at regular periods for 24 h, plasma is separated, and radioactivity levels of the residues may be measured. Main organs and tissues (lung, liver, kidney, heart, spleen, pancreas, brain, fat, and muscle) may also be collected, weighted and radioactivity may be measured [87, 92].

8.2.5 Changes in serum chemistry and cell type

Blood from the test animals may be collected for biochemical (triglyceride, cholesterol, glucose, glutamic oxaloacetic transaminase (GOT), and glutamic pyruvic transaminase (GPT)) and hematological investigations [88].

Chrishtop et al. [83], in their review on nanosafety versus nanotoxicology, in 2021, referred to studies that have demonstrated animal models with chronic diseases being sufficiently susceptible to nanomaterials even with low toxicity. Considering that chronic diseases, like bronchial asthma, are widely prevalent in various populations, they suggested that nanosafety should also be considered along with toxicity assessment of nanomaterials [83].

9. Conclusion

Techniques for evaluating the biocompatibility of nanostructured is not fully comprehensive since it is a continuously developing field where biomaterials are

being constantly developed for tissue engineering applications in regenerative medicine. Extensive use of nano biomaterials in regenerative medicine necessitates efficient techniques for evaluating toxicity at an affordable cost. Literature analysis clearly states that the cytocompatibility of biomaterials influences the output of in vitro experiments. The determination of parameters, such as cell type and nanoparticle concentration, required a detailed understanding of anticipated nanoparticle exposure and its metabolic activity within the human body. Hence selecting suitable methods for the analysis of in vitro toxicity will provide a proper idea about nanomaterials toxicity mechanism and can be effectively used in regenerative medicine.

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Conflict of interest

The authors declare no conflict of interest.

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