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Pathology

Nanoparticle-Induced Oxidative Stress in Human Cell Lines: Enzymatic Biomarkers and Gene Expression Disturbances

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Abstract | **Review Article**

Due to nanotechnology, there are new tools for delivering medication, carrying out diagnostics and treating diseases. One of its biggest issues is that nanoparticles (NPs) may cause oxidative stress which is linked to harmful effects on cells, inflammation and disease progression. It examines carefully the way engineered nanoparticles affect oxidative stress in human body cells, bringing updated information from nanotoxicology, molecular biology and bioanalysis. The first step is to classify nanoparticles as metallic, oxides and carbon types and note their physical and chemical characteristics, as well as the surface modifications needed for them to be taken in by cells and interact with them. HeLa, A549 and HepG2 cell lines which are derived from humans, are studied to determine if they represent disease changes and drug responses effectively. People pay special attention to the routes by which molecules are let into cells such as clathrin-mediated endocytosis and macropinocytosis and discuss how things like shape, size and charge impact localization and delivery of molecules. The basis of this review is to look at the mechanisms involved in oxidative stress when NPs are involved. Reactive oxygen species buildup, problems with mitochondrial function, lipid peroxidation and disturbance of redox homeostasis are important processes. We investigate if superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx) function as biomarkers that are affected by dose and time. We also look at changes in genetic and epigenetic factors, examples being damage to DNA, changes in gene expression in antioxidant pathways and reprogramming of the epigenomic code by methylation and microRNAs. Using analytical methods such as qRT-PCR, RNA sequencing, proteomics and ROS-detection assays helps diagnose effects of nanoparticle stress. We ultimately focus on how translational research can contribute by examining risk assessment, smarter NP design and better integration into the clinic. The review helps to organize knowledge by summarizing recent findings and possible future strategies which guide us in limiting nanotoxicity as we make full use of nanoparticles for human health treatments.

Keywords: Nanoparticles (NPs), Oxidative stress, Nanotoxicology, Reactive oxygen species (ROS), Cellular uptake mechanisms, Engineered nanomaterials, Biomarkers (SOD, CAT, GPx), Gene expression and epigenetics.

1. INTRODUCTION

Nanotechnology is now considered a leading field affecting underlying sciences, electronics, energy and especially biomedicine. One of the greatest contributions of nanomedicine is the creation of nanoparticles (NPs) which measure between 1 and 100 nanometers and have special features such as high surface area, exhibit quantum effects and can go through biological membranes. For this reason, nanomaterials can be used in many fields such as delivering medicine, imaging, diagnostics and treating diseases, particularly in oncology and precision medicine (Sanati *et al.,* 2022).

Distinct properties of biocompatibility, surface functionality and effectiveness benefit each type of class. Gold nanoparticles are used in both photothermal therapy and radiotherapy because they can be tweaked to alter the way they react to light (Prajapati & Bhattacharya, 2023) and polymeric NPs are now engineered to release drugs under specific conditions. Even so, nanoparticle-biological interactions can be tricky and sometimes harmful, mainly because of their effects on oxidative stress.

Oxidative stress is when the body makes more reactive oxygen species than it can handle with its antioxidants. Although ROS are needed for certain cell functions, an overload can cause protein, lipid and DNA damage that results in inflammation, cell death or tissue death. Researchers note that the large surface energy and reactivity of nanoparticles tend to increase oxidative stress. Budak (2019) reported that silver, silica and zinc oxide NPs caused increased levels of SOD, CAT and GS in HT-29 colon cancer cells, a response to stress. Workplace exposure to engineered NPs is linked to increased glutathione levels which indicates that the body responds to too many reactive oxygen compounds (Klusackova *et al.,* 2024).

Nanomedicine doesn't always see oxidative stress. They may use it for good effects too. In the right conditions, ROS can be used to help the body heal. Having a constant higher load of ROS, tumor cells are easily harmed by oxidative damage. Nanoparticle treatment for cancer seeks to make ROS inside cells grow too high in order to trigger cell death. Sanati et al. (2022) found that by injecting metallic and polymeric nanoparticles, doctors could raise ROS to improve chemotherapy, yet keep damage to healthy tissues reduced. Redox integrative therapies work by using pathways such as p53, MAPK and NF-κB to cause cancer cell death. Ways to measure oxidative stress in the body have been advanced with the use of nanotechnology. In 2019, Dalal and Biswas examined how nanoparticle-based fluorescent probes and imaging devices are used to detect reactive oxygen species in real-time. Having this function helps doctors find illnesses, judge how well medications work in the body and study how reactions occur in the body. Antioxidant capacity is seen in nanoparticles like cerium oxide (CeO_2) NPs. Their ability to produce and limit oxidative stress makes it important to think about how these particles are put to medical use. The key things that determine ROS generation are particle size, how they are shaped, their surface charge, their aggregation and how soluble they are. Being small, these particles are very reactive and can damage tissue by oxidation. Moreover, changes in oxygen, inflammatory reactions and metabolism within cells influence their reaction to nanomaterials. Low levels of exposure might cause antioxidant systems to strengthen, but high or prolonged contact can saturate these processes and lead to damage in cells or their DNA (Pelclova *et al.,* 2020).

As an answer to these concerns, recent research is centered on enhancing nanoparticles with smart features. They are designed to react to the high amount of reactive substances in tissue affected by disease. As an illustration, polymeric carriers that react to oxygen can deliver their drugs at raised ROS levels. More antioxidant-filled or antioxidant-coated NPs are now being designed to safeguard cells from oxidative damage, especially in cases of Alzheimer's, Parkinson's, ischemic injury and persistent swelling (as noted by Mauricio *et al.,*) The aim of this review is to look at the effects each has on the other in relation to oxidative stress throughout different areas of biomedicine. It reviews ways nanoparticles are carefully designed to target ROS generation, explains the different reactions in various cells and outlines new trends in medicine from the past five years. The idea is to create new medicines made from nanoparticles that target precision, are safe and effective by studying oxidative stress.

2. Classification and Properties of Engineered Nanoparticles:

2.1. Types of Nanoparticles (Metal, Metal Oxide, Carbon-Based, etc.)

Nanoparticles (NPs) are often grouped based on what they are made of and their inside structure. Figure 1 summarizes the types of engineered nanoparticles as metal-based, carbon-based, quantum dots, dendrimers and nanocomposites. Various chemical and physical features in classes of pharmaceutical drugs determine their relationships with living organisms and their various uses.

2.1.1. Metal-based nanoparticles

Additionally, metal-based nanoparticles include metal NPs, metal oxide NPs and binary oxide NPs. Au, Ag, Fe, Zn, Cu, Se and Sn are known to have metal nanoparticles because of their high surface-to-volume ratio, efficient catalytic properties and excellent electronics. On this account, they have practical value in antimicrobial coatings, biosensing, catalysis and drug delivery. A problem arises with silver and zinc nanoparticles because they can form harmful reactive oxygen species (ROS) that damage and kill living cells. Findings suggest that both Zn and Cu nanoparticles can be harmful to soil organisms and those found in water and when used in sunscreens, paints, wastewater treatment systems or food packaging, TiO_2, ZnO, MoO_3 and Fe_3O_4 metal oxide nanoparticles show semiconducting, photocatalytic and UV-blocking properties (Elmer *et al.,* 2018). These materials are toxic because of the ions they release, ROS they generate and disruption of cell membranes, with ZnO NPs being very harmful in in vitro experiments (Stuparu-Cretu *et al.,* 2023). Nanoparticles that contain binary oxides, for example Bi_2O_3, CeO_2 and CrO_2, show high redox properties and are mainly employed in catalysts and medical applications. Of particular note, CeO_2 nanoparticles scavenge free radicals and are investigated as potential anti-inflammatory substances.

2.1.2. Carbon-based nanoparticles

There are many types of carbon-based nanoparticles, for example, fullerenes, carbon nanotubes (CNTs), derivatives of graphene and carbon quantum dots. Chemicals such as C_{60} and C_{70} are spherical groups of carbon atoms widely employed in both drug delivery and imaging. Most nanoparticles are safe in the body, but their effects may change depending on how much is used (Wang *et al.,* 2019). Thanks to their outstanding tensile strength and conductivity, MWCNTs and SWCNTs are helpful in biosensors, storing energy and growing tissue. The amount of toxicity they display is determined by their size, surface modifications and how they group together and longer unchanged CNTs are usually more toxic (Sun *et al.,* 2021).

These materials in their quantum dot (QD) form

Researchers have found that CdSe, CdTe, ZnSe and Bi_2S_3 quantum dots change their light production as their size increases and this results in both strong photoluminescence and durability. These compounds find uses in bioimaging, LED technology and photovoltaics. Even so, many worry about their safety over time because they include heavy metals and do not easily break down (Devi, 2023).

2.1.3. DENDRIMERS

These nanoparticles, called dendrimers, have a tree structure and can be modified by altering the groups at the end of their branches. Internal spaces offer more room for drugs in nanoparticles which can be modified for increasing delivery to specific areas. Researchers now use dendrimers for cancer treatment and for delivering genes, although they may be more poisonous depending on the charge of their surface; cationic dendrimers tend to be the most toxic because they can affect cell membranes.

Mixing nanoparticles with other materials (ceramic, metal or polymer) in nanocomposites enhances their performance and expands their functionality. Al_2O_3/TiO_2 and Al_2O_3/SiO_2 ceramic matrix composites are found in high-temperature materials and also in protective coatings. Fe-MgO and Co/Cr nanocomposites are valued for increasing both strength and their magnetic behavior which makes them reasonable choices for catalysis and protecting against electromagnetic radiation. Nanocomposites with polymers such as those made from carbon nanotubes and titanium dioxide, are chosen for being both light and easy to shape, with uses in packaging, electronics and the auto sector. Engineered nanoparticles generally display enhanced thermal and mechanical properties because of how their components interact (Nikolaeva *et al.,* 2023). For example, metal oxide NPs and carbon-based NPs may have a lot of ecotoxic impacts. Treating a surface with carbon or adding polymers can either limit or boost the results shown by these effects (Yang & Luo, 2022). In addition, because composite systems involve a greater level of complexity, they present useful features as well as toxicity combinations that are being actively studied (Mujahid *et al.,* 2022).

All in all, Figure 1 outlines nanoparticles by their chemical features and shows how this relates to how they can be applied. Every class is defined by its own structures and features which determine how they act in medical, environmental and industrial areas. Between 2018 and 2025, studies have grown in number and improved our understanding of these materials, pointing to the need for strict control and adaptation to each application.

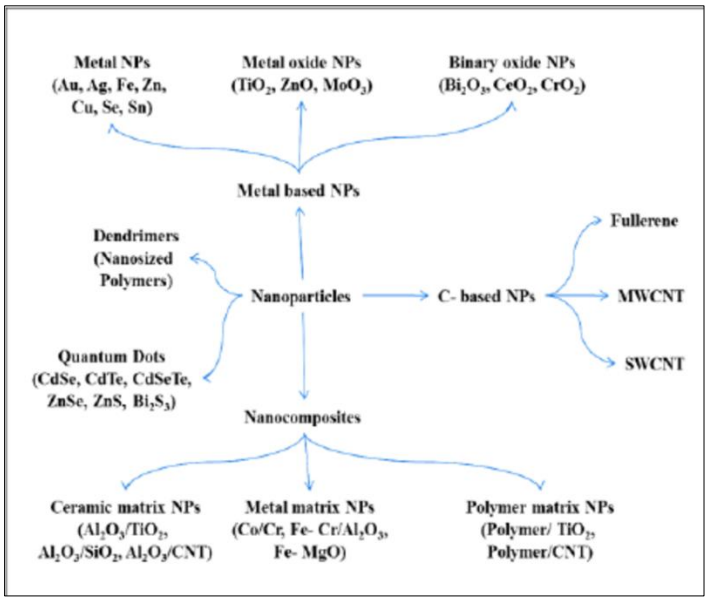


Figure 1: Classification of Engineered Nanoparticles Based on Composition and Structure

According to this figure, the common categories for engineered nanoparticles are metal nanoparticles (such as Ag or Au), metal oxide nanoparticles (such as ZnO or TiO_2), carbon-based nanoparticles (like fullerenes and graphene) and polymeric nanoparticles. Each form of lipids has its own physicochemical characteristics that influence their reactivity, stability and how they are used in biomedicine and industry.

2.2. Physicochemical Properties Influencing Biological Interactions

How nanoparticles behave in living organisms is largely guided by their physicochemical features. In Figure 2: Key Physicochemical Properties of Nanoparticles Affecting Biological Interactions, the main influences are size, shape, charge, coating, material and surroundings. Altogether, these different elements determine the interactions of nanoparticles with living things and affect both their safety and how well they work for medical purposes.

What matters most when it comes to biological interaction is the particle size. Nanoparticles usually fall between 1 and 100 nanometers and because of how small they are, they act differently from larger substances. In general, particles sized below 50 nm are well absorbed by cells using endocytosis and the smallest particles, those in the 20-30 nm range, are the favorite target of macrophages (Dhar *et al.,* 2020). Under 5 nm in size, particles might be cleared quickly by the kidneys and above 100 nm are much more likel Because of their size, nanoparticles smaller than 10 nm may move across biological barriers, including the blood-brain barrier which is useful for medicine but could also increase the possibility of toxic effects (Kapoor & Singh, 2021). Cellular interactions are influenced by the shape of nanoparticles as well. Compared to rod-shaped ones, spherical nanoparticles easily enter cells and because rods cover a larger surface, they may stay in contact with cells longer (Tejaswi *et al.,* 2020). Shapes such as nanoplatelets or nanostars show more surface area to interact with cell membranes but, at the same time, might affect how well synthesized nanomaterials get along with the blood (Arakha *et al.,* 2021).

Nanoparticle behavior in biological systems is largely influenced by their surface charge and what they are functionalized with. Cells usually absorb positive particles more effectively because they are attracted by the negative charge on the cell membrane, though such particles are often more dangerous for cells too (Dhar *et al.,* 2020). Differently, neutral or negative charged nanoparticles often have less damage to cells and thus stay longer in the body. Adding polyethylene glycol (PEG) or biological ligands, including antibodies or aptamers, improves the safety of nanoparticles, helps them bind to specific targets and decreases the chance of being eliminated by the immune system (Jaiswal *et al.,* 2022). In body fluids, nanoparticles tend to have a protective layer of proteins, called a protein corona which affects their identification, how they get taken up, how they circulate and their toxicity (Kamaly *et al.,* 2022; Forest, 2019). The makeup of a nanoparticle can strongly influence how it reacts to biological systems. Due to their strong structure, gold, silver, iron oxide or silica nanoparticles are regularly chosen for use in diagnostics, professional imaging and treating cancer. Liposomes and dendrimers among organic nanoparticles have a high level of biodegradability and produce low toxicity, though they can be unstable at physiological temperatures. Incorporating different materials such as inorganic, organic and carbon-metal mixtures into nanoparticles results in enhanced ways to guide them and time how much medication to send (Auría-Soro *et al.,* 2019).

Nanoparticle properties such as how their molecules are arranged, can also control how they work inside our bodies. Both the reactivity and distribution of aggregated particles usually change compared to their single-particle form. As an illustration, there are differences in the photocatalytic activities and toxic effects of the crystalline forms of titanium dioxide (called anatase and rutile) (Shende *et al.,* 2021). The behavior of nanoparticles can also be changed by pH, ionic strength and what is present in the body's fluids. The formation of protein corona is not the same for every serum; for example, fetal bovine serum and human plasma cause proteins to interact differently (Phogat *et al.,* 2018).

As a result, these physicochemical traits are important for biomedical purposes. Using nanoparticles of 50 to 100 nm and coated with PEG in cancer therapy allows them to use the EPR effect for passive build-up in tumor cells, according to Yagublu *et al.,* (2022). In the same way, using nanoparticles with selected ligands helps target diseased cells, cutting down the chances of unwanted effects elsewhere in the body. Knowing how nanoparticles function in the body is critical for making safe materials with clear effects when used. As found in Figure 2, the performance of nanoparticles in biological settings depends on size, shape, surface properties and what they are made of. When the key factors are understood, it becomes easier to design nanomaterials that are highly effective and have fewer adverse effects, supporting new advances in nanomedicine.

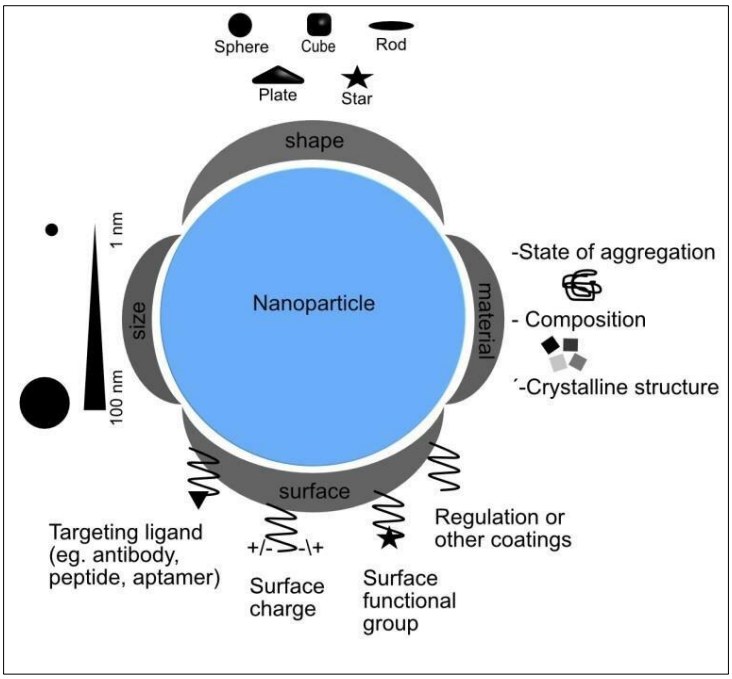


Figure 2: Key Physicochemical Properties of Nanoparticles Affecting Biological Interactions.

The schematic shows how nanoparticle specifics such as size, shape, surface charge and hydrophobicity determine their interactions with biological systems. Such features play a role in cellular absorption, movement through the body and toxic impacts, all of which affect the usefulness and safety of nanoparticle-based use.

2.3. Functionalization and Surface Chemistry for Targeted Applications

The enhancement of ENPs through surface treatments is key to recent developments in both biomedical and environmental nanotechnology because precision and safety matter most. When functionalization occurs, chemicals, polymers, ligands or biomolecules are purposely added along the surface of the nanoparticles. The steps make it possible for the nanoparticles to interact with targets such as diseased cells or pollutants and they also boost their solubility, how well they interact with the body and their stability in the system. The behavior of nanoparticles in living systems is mainly determined by charge, hydrophilicity and surface energy properties. Biological interactions involving nanoparticles are determined by their surface chemistry, including electrostatic attraction, bonding through hydrogen, weak van der Waals attraction and receptor-linked binding. Surface interactions regulate important aspects of interaction such as protein corona formation, how and where the drug reaches cells and how the immune system handles it. Researchers regularly use covalent and non-covalent strategies to customize how these interactions take place. Functionalization of nanoparticles with covalent bonds allows the attachment of PEG, folic acid or antibodies to their core, supporting steady delivery and aiming at a target population. By using opposite means compared to covalent methods, non-covalent strategies attach DNA, peptides or surfactants to materials via electrostatic interactions and the effects of hydrophobic molecules. Techniques for attaching or secreting drugs can change the efficiency of carrying drugs, the time nanoparticles spend circulating and their ability to avoid being detected in the body by the immune system, according to González-García *et al.,* (2022).

Using functionalized nanoparticles has brought major changes to drug delivery systems, making treatment more accurate, safer and more effective. Due to their adjustable size, interesting optical behavior and ease of changing their surface, gold nanoparticles are valuable for use in nanomedicine. The surfaces of NPs can be altered with PEG, antibodies, aptamers or chemotherapeutic drugs to allow release only in a targeted manner. Mugaka *et al.,* (2019) illustrated that coating AuNPs with PEG-COOH and PEG-biotin improved both their ability to carry cisplatin and their targeting ability. In addition, Nejati *et al.,* (2021) pointed out that gold nanoparticles functionalized with adequate ligands can improve tumor delivery and reduce adverse effects in photothermal therapy and molecular imaging.

Iron oxide-based magnetic nanoparticles (MNPs) are widely used both for cancer diagnosis and therapy. Because of their ability to move with external magnets, these particles are guided right to where you want them and their chemicals on the surface allow them to target and mix well with living tissue. The team led by Puja Gupta detailed how it is possible to construct MNPs out of polymers and tumor-targeted molecules that allow applications such as MRI and cell death due to heating (hyperthermia). The nanoparticles became much better at entering cells and remained longer inside tumor cells,

proving that how the surface is designed can directly influence treatment success. Among polymeric particles, those made with PLGA (poly(lactic-co-glycolic acid)) supply careful release and fast breakdown over time. By adding peptides or antibodies to their outerlayer, NPs can be more easily absorbed into cells and go to the organs they are designed for. Oliveira *et al.,* found that using cell-penetrating peptides along with PLGA nanoparticles in anticancer therapy helps increase the dose of the drug into tumor cells, boosts the drug's impact on cancer cells and improves survival. This demonstrates that chemically modifying outer surfaces is needed for smooth blood-stream travel and effective drug release into the cell as well.

Additional methods called LbL assembly have arisen as strong ways to coat nanoparticles with multiple functions. Using this method, layers of oppositely charged biomolecules are put on the nanoparticle surface, giving fine control over both their composition and the functions they perform. LbL coatings on nanoparticles were applied by Correa et al. (2020) to guide their behavior with ovarian cancer cells. Their testing found that a certain type of layered polyelectrolyte coating on nanoparticles greatly improved the targeting and entry of cells into them, highlighting how nanoparticle structure guides cell responses. On the other hand, hybrid and other pH, redox potential- or enzyme-responsive coatings can be used to control drug release only where needed which improves the accuracy of treatments. For nanoparticles to be useful in gene delivery, as vaccine carriers and for making biosensors, functionalization is essential. Incorporating polyethyleneimine (PEI) on the surface improves condensation and shipping of nucleic acids into the cell and when nanoparticles are conjugated with antibodies, they can target vaccine adjuvants. Specific biomarkers can be detected with high sensitivity and selectivity due to the use of aptamer- and enzyme-covered nanoparticles in biosensing. Because of their specific surface chemistry, these targeted applications allow nanoparticles to help in disease detection, healing organs and environmental checks apart from their medicinal work.

3. Human Cell Lines as Models for Nanotoxicology

3.1. Commonly Used Human Cell Lines (HeLa, HepG2, A549, etc.)

To test the biocompatibility, toxicity and workings of engineered nanoparticles (ENPs), human cell lines are important live models used outside the body. Despite being easily reproduced, stable genetically and responsive to nanomaterials, they are still main tools in nanotoxicology. Figure 3 shows how C-dots influence the numbers, structure and function of different cancer and non-cancer cell types. The authors look at the types of cell lines commonly used in studies on ENPs, their usefulness and the reactions observed after being exposed to these materials.

Researchers have been using HeLa cells, taken from cervical adenocarcinoma, for many years to better understand cancer. Because they can live forever, grow fast and are easy to look after, high-throughput toxicity testing is ideal for them. Scientists have used HeLa cells to measure the toxicity of nanoparticles made from gold, silver, graphene and carbon-based structures. In the dataset in Figure 3A, HeLa cell viability declined clearly when C-dot exposure was increased. In addition, Figure 3B indicates higher p53 protein levels, a classic sign of genotoxic stress and Figure 3C indicates that the Sub-G1 population rose, meaning that DNA has been fragmented by apoptosis. Results suggest that HeLa is well-suited for testing how nanoparticles can lead to oxidative stress and apoptosis by first triggering ROS included in mitochondrial pathways.

A549 cells which are from human alveolar basal epithelial carcinoma, are usually selected to reproduce the structure of lung tissue outside the body. They are designed to show type II pneumocyte features and are effective for evaluating exposure to airborne nanoparticles such as titanium dioxide, carbon nanotubes and metal oxides. As seen in Figure 3A, cells cultured in lower doses in the C-dot assay retained their vitality more, while higher doses caused viability to fall. It demonstrates that they are sensitive to the dose they receive which is important for studying lung toxicity in nanomaterial models. A549 cells are considered important because they react to nanoparticles with inflammation, problems in mitochondria and oxidative damage.

Scientists use HepG2 cells which originate from human hepatocellular carcinoma, to monitor hepatic metabolic pathways and toxicity. The cells can keep making plasma proteins and different cytochrome P450 enzymes which makes them useful for studying liver processes in the lab. Figure 3A demonstrates that HepG2 viability reduces, yet is still moderate, at the highest concentrations of C-dots. In addition, Figure 3B demonstrates an increase in p53, matching what is seen in response to DNA damage. Because of these traits, HepG2 cells can be used to predict how nanoparticles are metabolized, accumulated and removed from the liver in animals. Metabolicfunctions in these vertebrates add essential information concerning the body's response to nanoparticles and stress pathways. In order to understand cancer, MCF10A cells are important non-tumorigenic control cells for researchers. The gastrointestinal cells do not show any malignant potential and still look healthy, useful for testing the compatibility of possible new drugs. Figure 3A exhibits that MCF10A cells are much more resistant to nanotoxicity than their malignant partners. By comparing MCF10A to cancerous lines, researchers can measure how well and safely particular nanoparticle therapies would work.

Found in MD-A-MB-231 cells are no estrogen, no progesterone and no HER2 receptors which is crucial

in triple-negative breast cancer (TNBC). Because they are so invasive, these cells help determine how effective different anticancer nanoparticle treatments really are. From Figure 3A, the high concentration of C-dots caused the MDA-MB-231 cells to become much less viable, indicating a strong poisoning effect. There is also more p53 at high levels (Figure 3B) and a larger number of Sub-G1 cells (Figure 3C), reflecting apoptosis. Since they behave aggressively and their standard therapies do not work, they are valuable for looking at ROS, damage to mitochondrial membranes and nanoparticles' impact on the cell's inner skeleton.

The trends in the figure series reinforce the fact that models are diverse. Results in Figure 3A show that cancer cells HeLa and MDA-MB-231 are affected more than MCF10A and A549 by C-dots, possibly indicating that cancer cells are more susceptible to changes caused by certain nanoparticles. Figure 3B adds to this by demonstrating that increased p53 expression in stressed cells signals DNA damage and apoptosis. Analyzing the cell cycle from Figure 3C, we find that apoptosis, particularly malignant, is present by the higher frequency of Sub-G1 phase which means DNA is fragmented.

Many more types of human cell lines contribute importantly to the testing of nanotoxicology. In order to test nanoparticle interaction, MCF-7 cells are commonly used as an example of estrogen-regulated breast cancer. HT-29 and HCT116 cells are frequently employed in gastrointestinal toxicity studies involving colorectal adenocarcinoma. The U87 and SH-SY5Y cell lines are essential for studying neurotoxicity and ability to breach the blood-brain barrier. In addition, fibroblast (3T3) and endothelial (HUVEC) lines are employed in experiments involving nanoparticles in the dermal and vascular environments. They allow researchers to look at nanotoxicity in different organs of the body. Overall, HeLa, A549, HepG2, MCF10A and MDA-MB-231 human cell lines serve as vital models for exploring the biological and molecular changes brought by engineered nanoparticles. Because of their special traits, experts are able to analyze how cells become toxic, undergo genetic changes, experience oxidative stress or enter apoptosis. As seen in Figure 3 and represented by recent literature, multi-cell line testing is important for assessing nanoparticle safety and creating new medical treatments.

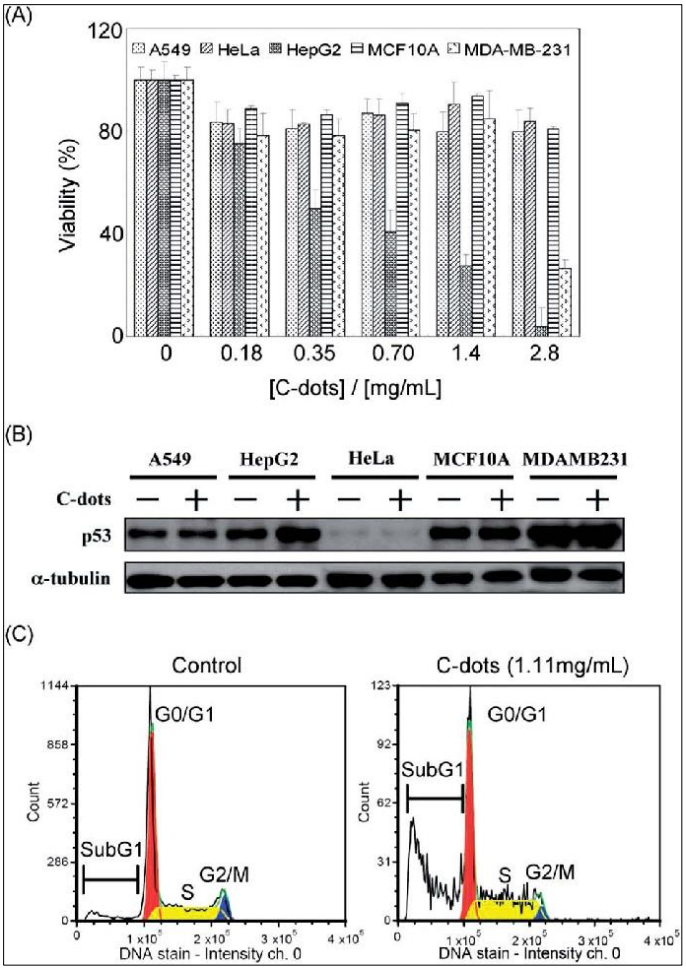


Figure 3: Commonly Used Human Cell Lines in Nanotoxicology Studies

Here you can see that most nanotoxicology studies use HeLa (cervical cancer), HepG2 (liver carcinoma), A549 (lung epithelial) and BEAS-2B (bronchial epithelial) cells. Cells are related to the tissues

they are part of and the main pathways they come into contact with nanoparticles. They are widely used in studies on how nanoparticles impact cytotoxicity, oxidative stress and inflammation, so they are important models for ensuring nanoparticles are safe for use.

3.2. Relevance to Human Physiology and Disease Models

For a long time, A549, HeLa and HepG2 have been important in nanotoxicology research, mainly because they can stand in for some areas of human physiology and disease. With nanotechnology being used more in biomedical, pharmaceutical and industrial settings, finding testing that is both biologically accurate and ethical is now necessary. Human cell lines provide a way for scientists to reliably study the effects of nanoparticles, focus on their uptake into cells and see their distribution inside the cells, all in a way that represents human tissue behavior.

Figure 4 demonstrates the changes in nanoparticle actions based on the type of culture system they are in. A549 lung epithelial cells are portrayed under exposure to silica nanoparticles in two culture environments: the standard flat glass system and the physiological Transwell membrane setup. The way cells respond to nanoparticles, shown in panels e and f, suggests that the way materials are arranged in 3D cell culture can have a big impact and supports the need for models that mimic the body's environment better.

While human cell lines are not diverse because they are monoclonal and immortal, they keep a lot of their own tissue's physiological characteristics which adds value to their use in screening toxins in specific organs. A549 cells which are obtained from the alveolar type II cells of humans, are often employed to stand in for lung cell barriers and for study of the effects of inhaled substances. HepG2 cells, since they remain metabolically active and can handle foreign compounds, are fitted for toxicity and metabolism tests involving hepatic nanoparticles. Since HeLa cells, taken from cervical cancer, reproduce quickly and are well understood genetically, researchers use them often for cytotoxicity screenings. These models fail to mimic how the body's systems such as the vascular and immune systems, work, yet combining them with macrophages and using organ-on-chip platforms or Transwell membranes has raised their biological significance. The accuracy of nanotoxicity research has improved with the use of new in vitro systems such as Transwell and air–liquid interface (ALI) cultures. When cultured on Transwell inserts, the nanoparticles in A549 cells were more easily taken up than those in cells grown on flat glass, as the numbers strongly suggested (X = 20). This result shows that accurate 3D design and conditions of testing are required in in vitro models. Tissue cultures made of both epithelial and immune cells are better able to respond in ways that are similar to in vivo tissue reactions. As a case in point, Zhang et al. (2019) found that combining epithelial cells with macrophages allows a better detection of inflammation and membrane leakage caused by nanoparticles.

There has been ongoing progress in making organ-specific in vitro models smarter and more useful. The use of epithelial-endothelial co-cultures in pulmonary models improves the simulation of the lung barrier and allows us to predict nanoparticle translocation, inflammation and the immune reaction more reliably. By using HepG2 or differentiated HepaRG cells, scientists can learn about how nanoparticles metabolize, detoxify and build up in the liver. Investigators have found that in 3D liver models, nanoparticles are sorted in a distribution that mimics the zoned structure seen within the liver in animals (Böhmert *et al.,* 2018). Evidence in neurotoxicity tests reveals that hNLCs, made from human mesenchymal stem cells, respond differently to magnetite nanoparticles depending on the dose (De Simone *et al.,* 2019), showing that modelling the blood-brain barrier and how neurons are impacted is not simple.

Caco-2 cells are considered the best model for evaluating function of the intestinal barrier. Examining ZnO nanoparticles, researchers found that nutrient transport was disrupted, certain transporter gene activity was altered and microvilli were damaged, similar to effects observed in the real gastrointestinal tract (Moreno-Olivas *et al.,* 2019). Besides being used to examine toxicity, human cell lines are used to simulate various diseases. Researchers use such cell lines MDA-MB-231, HT-29 and U87 to examine nanoparticle absorption by cancers, the response that occurs and the way drugs are delivered. By growing epithelial cells and THP-1-derived macrophages in a mixed culture, we are able to examine how nanoparticles influence both cytokine secretion and immune activation closely. Researchers have built pulmonary hypertension models by stretching cardiac endothelial cells, using them in studies to examine how nanoparticles interact in diseases with oxidative stress and low levels of nitric oxide (Deweirdt *et al.,* 2021).

No matter the improvements, traditional monolayer cells do not completely represent the complex and alive state of actual human tissues. Many nanoparticles respond differently to in vivo conditions that include fluid flow and differences in oxygen and mechanical stress which are not fully coped with in fixed experiments in vitro. For this reason, scientists now use 3D spheroid cultures, organ-on-chip microfluidic devices and perfusion bioreactors, each of which attempt to recreate the organ level in experiments (Maia *et al.,* 2020). They enhance the prediction of nanoparticle behavior and the risks it may pose to humans.

In addition, diversity in nanoparticle dosage between experiments in dishes or animals is a hurdle. Research in this area has shown that how nanoparticles

dissolve, their charge on the surface and their tendency to clump together can impact both dangerous exposure and a particle's toxicity. It is essential to bridge this gap for both creating regulations and designing medicines (Smith & Skinner, 2021). Overall, human cell lines help to unite the main principles of cellular effects with how these might apply in real people. Advanced culture models, combined culture and systems for particular tissues increase their usefulness. According to Figure 4, how well the information will translate and the accuracy of the findings depend greatly on model selection and experimental planning. In the future, adjusting human cell models to resemble actual tissues and diseases will help eliminate hazards from nanomaterial applications in medicine and industry.

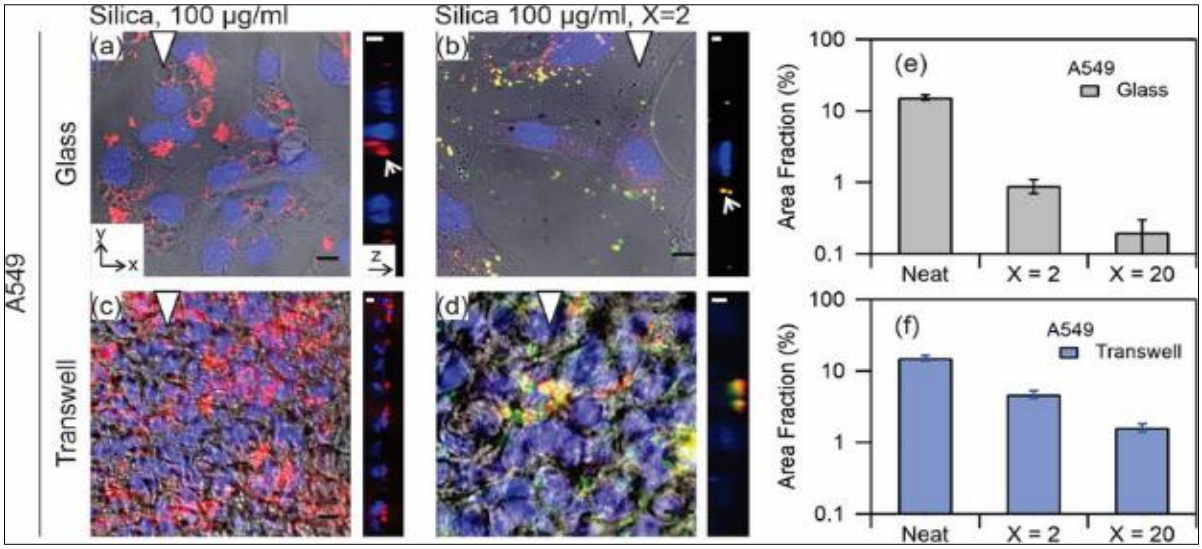


Figure 4: Relevance of Human Cell Lines to Physiology and Disease Modeling

The figure compares the toxicity of silica nanoparticles (SiO_2 NPs) on cancerous (A549 and HepG2) and normal (NHBE and HH) types of human cells. The study examines if the cells are alive, how they die and how many nanoparticles are inside them, showing that the cell lines respond to nanoparticles much as the corresponding tissues do. They help clarify the use of these models in studies of how nanoparticles impact the body.

3.3. Advantages and Limitations in Nanotoxicity Assessment

One of the main ways nanotoxicology proves itself useful is by testing in vitro to better understand the toxic effects, how cells take in nanomaterials and their biocompatibility. As for these models, human cell lines are widely used because they are ethical, can be used in large studies and are reproducible. Nanoparticles were tested on these cells to check for cytotoxicity, oxidative stress, risks to genes and other effects which helped shape the early safety profiles of nanomaterials. Still, although using human cell lines simplifies the settings for experiments and improves the human relevance, their predictions for complex living situations are constrained. Commonly used human cell lines such as A549 (alveolar epithelial), HepG2 (hepatic carcinoma), HeLa (cervical carcinoma) and THP-1 (monocyte-derived macrophage), are easy to use and cost-effective, making them perfect for screening large amounts of nanoparticles. Since these living lines are commercially available and easy to culture, researchers have an economical way to study different nanomaterial amounts, sizes, coatings and time of exposure (Kim *et al.,* 2022). Since they come from humans, their models can avoid the metabolic differences that appear between humans and animals. It has been found that HepG2 cells' reactions to substances are similar to those of hepatocytes, so these cells are used for checking the metabolism and removal of nanomaterials by the liver (Moghimi *et al.,* 2019).

The possibility of people repeating the findings is another strong point. Because of their consistent, unchanging genes and capacity to be used indefinitely, human cell lines give researchers the same outcomes in every sample and lab. Having similarity is crucial when comparing different toxic effects and when trying to find slight differences in nanoparticle coatings or how easily they mix in fluids (Gholizadeh-Ghaleh Aziz *et al.,* 2019). They have made it possible to probe pathways that produce reactive oxygen species (ROS), harm mitochondria, lead to autophagy, cause DNA damage and activate apoptotic signals. Szwed et al. (2019) presented that small changes in nanoparticle coatings could cause different stress responses in A549 cells, suggesting how sensitive in vitro platforms are for understanding mechanisms.

Since the appearance of modern analytical systems, people have used human cell lines in nanotoxicology more frequently. With single-cell methods like sc-ICP-TOFMS, one can understand how nanoparticles are distributed in individual cells such as

A549 and THP-1 cells, something that is hidden in bulk studies (Hendriks *et al.,* 2023). Human cell lines also help research follow the ethical goal of using animals less which fits with the 3Rs (Replacement, Reduction, Refinement) principle. A number of authorities are now considering validated in vitro methods in the assessment of safety for nanomedicine and related products (Sri *et al.,* 2021).

By having these positive points, human cell lines also present core reasons for concern. A monolayer of cells does not show the three-dimensional (3D) design seen in tissues or the matrix and multiple cell types found in organs. According to Choi *et al.,* (2021), using 2D and 3D HepG2 cultures found that silica nanoparticles impact the cells differently, suggesting that extracellular matrix and cell-cell interactions are important in influencing how cells respond. One more disadvantage is that immortalization can bring about changes in the way genes and proteins are expressed. Changes can cause difficulties with cellular intake; alter the normal way cells use energy and change stress responses. In their study, Busch *et al.,* (2023) explained that difference in transporter expression and metabolism of nanoparticles between immortalized cell lines and primary cells may make prediction of nanoparticle bioavailability inaccurate.

Also, they cannot provide much detail about immune system functions. The typical lines often omit important things such as cells that present antigens and immune effectors producing cytokines. While THP-1 cells help understand partly how macrophages respond, they are not a perfect representation of how innate immunity truly works. This makes it more difficult to examine the effects of inflammation or immunity after someone is exposed to nanoparticles (Verdon *et al.,* 2020). Response to the same nanomaterials by cells from similar tissues may not be the same. Based on a study done by Nezhad *et al.,* (2022), carbon nanotubes act differently on various types of epithelial and endothelial cells, showing that not one cell type can accurately represent possible reactions to all kinds of nanoparticles.

The fact that cells vary in their endocytic and phagocytic activity means that nanoparticle uptake and where they go inside cells is uneven across different cell lines. Kim *et al.,* (2022) show that, unlike primary cells, cancer-derived cell lines like HepG2 and A549 take up fewer nanoparticles which leads to an underestimation of both the reach and effects of these nanoparticles. Besides, measures related to the whole body such as the accumulation of drugs in the liver or other organs, cannot be directly observed with static in vitro systems. Using in vitro approaches alone are not useful, according to Sarma *et al.,* (2021). This is why it's important to use data from in vivo and organ-on-chip assays.

In order to fix these limitations, advanced testing systems outside the body are being created. Such systems are closer to the natural environment because they include the proper cell organization, nutrient gradients and matrix. Gholizadeh-Ghaleh Aziz and his colleagues (2019) stated that 3D stem cell cultures with added nanoparticles were more useful for predicting the toxic effects in tissue engineering applications. Organ-on-chip technologies copy movement (shear stress), blood flow (perfusion) and interactions between organs by using microfluidic channels. In their research, Busch *et al.,* (2023) applied simulated platforms to study both hepatic and pulmonary scenarios, making their predictions about nanoparticles more precise.

Advancements have occurred recently with the use of high-content screening and label-free cytotoxicity techniques that avoid problems related to the optical qualities of nanoparticles. By using methods like impedance-based monitoring and colony formation assays, you can get more accurate information about cell viability and how cells work (Won *et al.,* 2022). Also, using techniques like transcriptomics, proteomics and metabolomics permits a thorough assessment of nanoparticle effects on cellular pathways and metabolism. In their study, Xu *et al.,* (2023) proved that using omics integration can sort nanoparticles by their ways of working, leading to more informed development of safer nanomaterials. Case studies help explain both the problems and the modern solutions that are being developed. Choi *et al.,* (2021) reported that both the type of scaffold and the serum composition strongly affect silica nanoparticle toxicity to HepG2 cells in 3D settings, as confirmed in their experiment. As cited by Rubio *et al.,* (2020), polystyrene nanoplastics brought about DNA damage specific to cell type in THP-1, Raji-B and TK6 hematopoietic cells, showing that using the correct model is pivotal for toxicological assessments. In their research, Chatzimitakos *et al.,* (2018) saw that using carbon nanodots helped the growth of HEK-293 cells only when certain conditions were met, hinting that there are circumstances where nanomaterials could offer benefits.

All in all, human cell lines are key for preliminary testing, but they should not be considered a perfect representation of processes inside the body. Advances in 3D co-cultures, organ-on-chip and new forms of data analytics are needed to unite basic science and medical uses. Using a combination of laboratory, computer and animal testing is necessary for effective evaluation of risk and development of effective nanotherapeutics for clinical use.

4. Cellular Uptake and Intracellular Trafficking of Nanoparticle

4.1. Endocytosis Pathways and Uptake Mechanisms:

It is very important to understand cell uptake of nanoparticles (NPs) in order to make safe and effective nanomedicines. As is shown in Figure 5, cells mainly take up nanoparticles by undergoing endocytosis and related processes, for example, clathrin-mediated

endocytosis, caveolae-mediated endocytosis, macropinocytosis, phagocytosis and pinocytosis. The fate, how well the particles work as therapies and possible toxic effects depend on the route each system uses. The clathrin-mediated endocytosis (CME) pathway is the one that is best understood and used the most. In this process, clathrin triskelions are assembled into a lattice in the inner part of the plasma membrane and this causes the formation of vesicles that pick up extracellular material. Nanoparticles between 50 and 200 nanometers generally pass into the cell in this way because these are the ideal sizes for clathrin-coated vesicles. According to Li *et al.,* (2023), wrapping spherical nanoparticles is more effective because of their symmetrical form and minimal impact on the membrane, compared to wrapping the others. So, CME helps make CME an attractive target for drugs that must get into the cytoplasm after entering the cell.

Another lipid raft–related mechanism is called caveolae-mediated endocytosis (CvME) which happens when caveolin-containing flask-shaped invaginations of 50–80 nm serve to take up particles from the plasma membrane. Rather than CME, caveolae-mediated uptake goes to caveosomes or the endoplasmic reticulum instead of being processed in lysosomes. It is particularly helpful in maintaining the correctness of therapeutics. They showed that the stiffness of the substrate affects the movement of particles into cancer cells through both CME and CvME, showing that both CME and CvME are sensitive to changes in substrate stiffness (Wei *et al.,* 2019). Even so, CvME mainly affects certain cell types and is less important for other epithelial cells, so it cannot be used everywhere.

Cells use macropinocytosis to pull in large amounts of fluid and particles by slowly extending their membranes with the help of actin which is suitable for nanoparticles that are 200 nm or larger. It is commonly more active in cancer cells and cells of the immune system. Then, machinery assembles to seal off the membrane as a macropinosome which later fuses with lysosomes. According to Means et al. (2021), both the shape and the coating of nanoparticles may affect how much macropinocytosis occurs, especially in highly active or cancerous cells. It is beneficial for treatment of cancers that grow by taking in lots of nutrients through macropinocytosis, a process for gathering nutrients.Because phagocytosis is limited to immune cells, it has a more restricted use than leukocyte trafficking, for example. Using receptors, cells change their interior structure to enclose big particles into phagosomes. As this process is selective and specialized, it is rarely present in non

immune cells, though it is important for measuring side effects of immune reactions and removal of nanocarriers. Much like macropinocytosis, pinocytosis allows cells to nonspecifically take in fluids and solutes from outside the cell. It is less involved than other types of endocytosis in getting nanoparticles into cells. Sometimes, nanoparticles can diffuse straight across the cell membrane if they are coated with specialized peptides called cell penetrators.

Nanoparticles move safely long internal routes within a cell once they are taken in. As picture in Figure 5 reveals, they initially enter early endosomes and it is here where the first sorting stage occurs. Things that need to be broken down are sent to late endosomes and eventually unite with lysosomes. For medicines that are halted by acid in lysosomes, getting out of the endosome is very important. It is also possible for nanoparticles to exit the cell either back to the environment outside or over to another cell. The authors of this study (Adinolfi *et al.,* 2018) found that polymeric nanoparticles were partially removed from A549 epithelial cells by lysosomal exocytosis after initially entering using endocytosis.

Nanoparticle traits including size, shape, charge and stiffness play a major role in choosing how cells take them up. Microvilli usually pick up small particles (10–100 nm) by CME or CvME, whereas for large particles (>200 nm), macropinocytosis is typically the method chosen (Varma *et al.,* 2021). Nanoparticles that are rod-shaped or star-shaped impact membranes differently. Thamizhchelvan *et al.,* (2024) found that iron oxide nanorods were more readily taken up by cancer cells because they line up with phagocytic and clathrin-mediated mechanisms. Surface charge is yet another factor; positively charged nanoparticles are more likely to be taken in by negatively charged cell membranes mainly via CME and macropinocytosis (Xiao *et al.,* 2020). Protein corona formation (where proteins in blood coat the nanoparticles) can lead to shifts in cellular uptake, causing it to happen through macropinocytosis rather than typical receptor-mediated endocytosis (Ding *et al.,* 2018).

Where a cell is located matters as much as its function. Different cells favor different ways of endocytosis. A549 epithelial cells mostly go for CME and CvME, while macrophages rely on phagocytosis and macropinocytosis. Depending on what they need, cancer cells make use of different ways to take in substances. Cellular choices are strongly guided by external factors such as the stiffness of their environment, the serum they are in and the oxygen available, according to a recent study (Rennick *et al.,* 2021).

A number of approaches are taken to study and divide complex processes into smaller components. Chemical inhibitors like chlorpromazine (CME), filipin (CvME) and cytochalasin D (macropinocytosis) assist in sorting out particular biological processes. Tests carried out with siRNA or CRISPR tools help knock down important proteins such as clathrin, caveolin and dynamin. Imaging tools such as confocal microscopy and TEM show nanoparticle uptake and SERS allows for

understanding nanoparticle uptake routes without adding labels (Yılmaz & Çulha, 2021). Still, it is important to consider that chemical inhibitors can affect things apart from what they are intended for and experiments may give different results with different types of cells and types of nanoparticles used (Rennick *et al.,* 2021). Finally, endocytosis is the main way nanoparticles and cells communicate. Each of these paths is different and the effects they have are driven by things like the nanoparticle features and type of cell. It is important to fully understand these processes to make targeted nanomedicines, reduce side effects and improve delivery success. Figure 5 shows how different processes are linked and important within the cell.

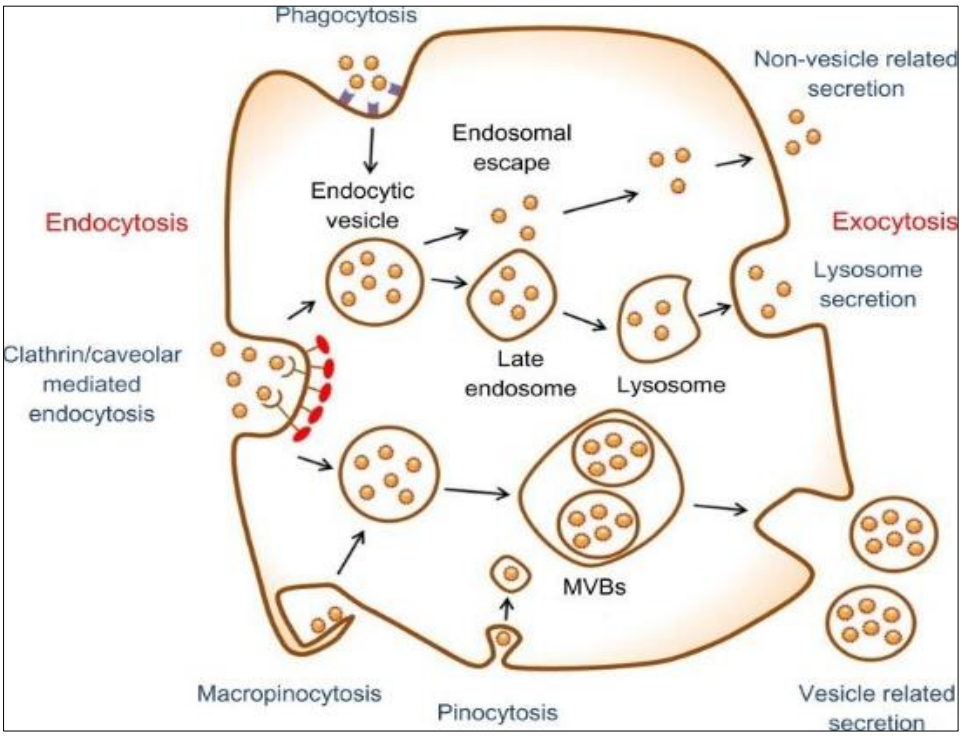


Figure5: Schematic of Endocytosis and Exocytosis Patterns of Nanoparticles

Various forms of endocytosis, including with clathrin, caveolae and macropinocytosis, how nanoparticles enter the cells. They get transferred into the cell using vesicles and their interactions with different parts of the cell decide whether they will be released using vessels or directly.

4.2. Subcellular Localization and Fate of Nanoparticles

Results from subcellular trafficking and fate of NPs are important to their usefulness, how compatible they are with the body and the risks of damaging cells. Once inside the cell, via the mechanism of endocytosis (Figure 5), nanoparticles are processed inside the cell and placed in different sections, where they might be degraded, stated in the cell or leave for the cytoplasm or nucleus. All the steps are easier to follow in Figure 6 which depicts the endosomal–lysosomal pathway by tracing antisense oligonucleotides (ASOs) as an example of cargo. It illustrates how particles pass through early endosomes, late endosomes (LE), multivesicular bodies (MVBs) and lysosomes and describes the various ways they may exit such as back through early endosomes, to the Golgi or ER or by modulating the membrane.

Once nanoparticles enter cells, they often move to early endosomes (EE) which play a major role in their sorting. Endosomal escape allows some nanoparticles to come out of the vesicles and enter the cytoplasm which is necessary for drugs meant to work in the cytosol or nucleus. The pathway involves bending of membranes, forming of vesicles and the activity of special sorting proteins, including annexin A2 (ANXA2). At this point, vesicles may go on to become late endosomes or can travel back to either the plasma membrane or the Golgi apparatus. With the help of super-resolution microscopy and correlative light-electron microscopy (CLEM), researchers can now closely examine nanoparticles in these compartments. Chemicals such as chloroquine can weaken the vesicle membrane, helping the molecule escape the endosomes and get into the cell by all routes.

While nanoparticles go through late endosomes, they start to have a higher chance of being broken down in lysosomes. Late endosomes must check an item's safety status and then usually change into MVBs before uniting with lysosomes. In lysosomes, the environment becomes very acidic which favors the break-down of nanoparticle based products that use biodegradable polymers, liposomes or some metals. During this stage, the way nanoparticles behave is often made to respond to

pH levels; for example, the presence of dimercaptosuccinic acid (DMSA) on iron oxide nanoparticles results in their lysosomal uptake and the ability to start autophagy through acidic substances produced. As a result, lysosomal entrapment might limit delivery or allow controlled release depending on what the nanoparticle was made for.

The way to effective cytoplasmic delivery for nanoparticles is often to stop them from entering the endosome and being sent to the lysosome. Among the strategies used are disruption of membranes by pH-sensitive polymers, direct fusion or formation of pores with the help of functional proteins and "proton sponge" action performed by certain cationic polymers like polyethyleneimine (PEI). Experiments using the SNAPSwitch assay highlighted that the way endosomes are entered can affect how well nano drugs escape the lysosomes which is why designing nanoparticles with care is important for optimal treatment results.

Some nanoparticles take a reverse pathway and are sent to organelles further inside the cell such as the Golgi apparatus or the endoplasmic reticulum (ER). This type of trafficking happens through vesicles called COPII that carry the mannose-6-phosphate receptors (M6PR) from the TGN to the ER. This method is especially needed for the delivery of protein and gene therapies that must get into the nucleus. Bailey-Hytholt *et al.,* (2023) noted that reaching gene targets with CRISPR and antisense systems usually requires using these pathways.

Gaining access to the nucleus is more difficult. Nanoparticles are either carried through the cytoplasm by bypassing the barriers or delivered via traffic through vesicles linked to the ER to get to the nuclear envelope. Putting NLS on nanoparticles helps the particles enter the nucleus by using the nuclear pores. Using ferritin to create protein particles leads to improved targeting of these particles to nuclear areas, changing gene expression in immunesection, causing a change in expression particularly seen in RAW264.7 macrophages. As soon as nanoparticles are inside specific cellular structures, some changes may happen in the form of biotransformation. Examples are chemical or enzymatic breakdown that usually takes place in lysosomes or MVBs. If nanoparticles are unable to leave the body or their clearance is delayed, autophagy might be activated and compel stress reactions. Besides, special coatings for nanoparticles enabled by pH or enzymes provide a targeted way to release therapeutics. For example, over time, gold nanoparticles (AuNPs) move into lysosomes, disrupt the shape of these vesicles and are flushed out by the liver. As time matters here, developing drugs requires careful attention to the stability and degradation of nanoparticles.

All nanoparticles do not stay within the cell forever. Exocytosis helps move things out of a cell or between cells. Release of drugs by vesicles or direct, non-vesicle flipping of the membrane may help control the drug's interaction in the body and the length of its effectiveness. Synthetic smectite nanoclays used in bone regeneration were found to be released from stromal cells through lysosome-associated exocytosis which supports their use in different treatments. After being internalized, the protein layer around nanoparticles, called the intracellular protein corona (IPC), influences their fate. How IPCs form can influence nanoparticles to attach to particular organelles in the cell, for example mitochondria, lysosomes or the ER. This corona also takes part in influencing how cells react to stress. It was determined in a proteomic study that the transportation of gold nanoparticles by Caco-2 cells depends on the IPC structure which is linked to specific accumulation in various organelles and how they respond biologically.

Imaging and tracking tools have made it much easier for scientists to follow nanoparticles within cells. It is possible to see both the location and function of molecules with dSTORM and TEM at resolution below that of organelles. Label-free techniques have shown polystyrene and MoS_2 nanoparticles in different organelles and with magnetic recovery methods, organelle-targeted purification and examining cells after exposure are both successful. They explain how nanoparticle structure, their chemistry and how they are designed determine their behavior and outcomes. Ultimately, nanoparticles are handled within cells by traveling through endosomes, staying in certain locations, exiting if necessary, being destroyed and sometimes coming out of the cell by exocytosis. The cell's response and how successful the therapy is depend on where the nanoparticles end up which could be the lysosome, nucleus, Golgi apparatus or ER. Understanding the pathways, as seen in Figure 6, is very important for creating safer, more effective and organelle-targeted nanomedicines.

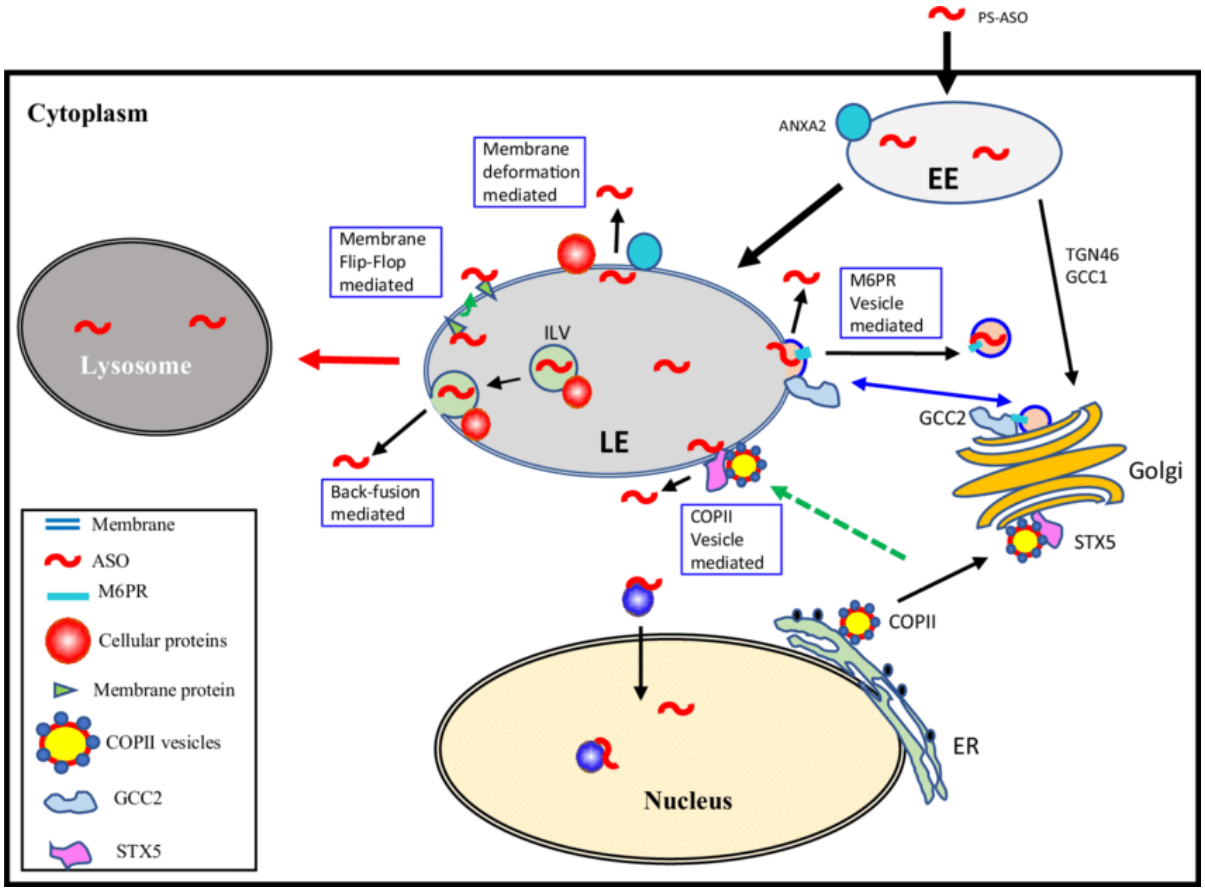


Figure6: Subcellular Trafficking and Fate of Internalized Nanoparticles

It illustrates how nanoparticles behave within a cell after endocytosis and how they may travel into early and late endosomes, lysosomes, the Golgi complex and may be found in both the nucleus and the cytosol. This area of research covers how changing the surface of particles helps them get out of endosomes or activate autophagy which can influence drug effectiveness and where it stays in the cell.

4.3. Influence of Particle Size, Shape, and Surface Charge:

Nanoparticle (NP) characteristics, especially their size, shape and electrical charge, greatly influence how they affect cells, are processed by the body, distributed throughout the body, removed and whether they work as expected. As Figure 7 illustrates, the characteristics of these particles help decide how macrophages respond to them which can impact the overall health of the body, the immune system and the effectiveness of therapy. Working from 2018 to 2025, contemporary scientists have found interesting insights into how both parameters impact nanomedicine and tests for toxicity. Nanoparticle size is very important in controlling how nanoparticles affect and move within cells. It is mainly through the interaction between nanoparticles and the cell membrane that internalization of nanoparticles is made possible. Empirical evidence demonstrates that endocytosis happens most effectively with particles measured in the 10–100 nm size range. According to Sharma *et al.,* (2019), nanoparticles sized between 10 and 60 nm showed the highest uptake in cell culture studies, whereas larger nanoparticles (over 200 nm) tended to be cleared by macrophages. When particles have diameters of 100 nm or less, the EPR effect enables them to seep through permeable tumor vessels and be retained at the tumor site, but larger particles usually end up in the liver and spleen. Besides, different-sized nanoparticles behave differently: really small nanoparticles are filtered out quickly, while those in the middle range linger and gather mainly in tumors. Alternatively, particles over 200 nm are absorbed by the liver and spleen after being recognized and removed by the mononuclear phagocyte system which is explained by Ledford and coworkers (2023).

Shape of particles is an important factor in determining which cells take them in and how they are distributed throughout the body. The way nanoparticles are designed geometrically decides their ability to stick to tissues, how long they stay inside the body and the rate at which they enter cells. Due to their easier wrapping by the cell membrane, spherical nanoparticles are often consumed more rapidly by cells, whereas rod-shaped particles may take a little longer to enter the cell and can deliver drugs more precisely which is useful in particular applications. Through their disc shape, nanoparticles bubble along and drift between nearby blood vessels, increasing their chances to stick on the endothelial wall.

It was reported by Chauhan *et al.,* (2021) that nanoparticles with a disc-like shape mostly collect in areas with lots of blood vessels which supports local delivery. Also, in dynamic shear streams, rod- and disc-shaped nanoparticles increase their adhesion to endothelial cells which helps them remain at specific sites. According to Guo *et al.,* (2018), nanosheets carrying docetaxel work better and stay longer in the tumor than the round versions. It was shown by Cybulski *et al.,* (2024) that spherical particles could pass deep into 3D tumor models and contrastingly, rod-like particles mainly gathered near the edge of the tumors.Nano–cell interactions largely depend on the surface charge or zeta potential, of nanoparticles. Cationic nanoparticles are quickly taken in by cells because of their attraction to the negatively charged phospholipid bilayer covering the cells. Anionic particles enter cells less well, but they are preferred because they are less likely to cause an immunological reaction and because they adsorb less nonspecific protein. Because neutral nanoparticles hardly stick to cell surfaces, they are well-suited for stealth systems to ensure the drug stays in the blood for a long time and to avoid recognition by the immune system. According to Jeon et al. (2018) and Vo et al. (2024), the charge on the surface of microparticles matters for deciding how cells take them up and it also affects the microparticles' distribution and clearance in the body. It is apparent from Figure 7 that macrophages actively take up both highly cationic and highly anionic nanoparticles because of strong electrostatic forces which can hasten their clearance and may influence the degree of inflammatory responses based on the surface properties of the particles.

Particle size and surface charge together shape a substance's absorption, distribution, metabolism and the effect on the body. According to Sodipo and Mohammed (2024), adding zwitterionic coatings to gold nanoparticles (GNPs) of 50 nm substantially slowed their removal by the immune system and enhanced their blood circulation time. In the same way, Du et al. (2018) found that positively charged polymeric nanoparticles had higher absorption from the oral cavity and better movement across Caco-2 intestinal cells, pointing to how optimizing charge affects both bioavailability and passing through epithelial barriers.

The way atoms are shaped and charged is very important too. Vo and his colleagues synthesized single-chain nanoparticles that look like tadpoles and have moderate, positive charges (around 15 mol%) which led to the best cellular uptake without causing too much damage. When cationic groups were applied too strongly, it disrupted the membrane, meaning charge must be adjusted together with shape to ensure effectiveness and safety.Rapidly, proteins combine in the corona layer on nanoparticles which can change the nanoparticles' size, surface qualities and effect on cells. It greatly affects both the way substances are taken up and the way they are transported inside the cell. Based on Ding et al.'s (2018) research, protein corona can change the way cells take up nanoparticles, affecting what happens to them after internalization. Attaching PEG or zwitterionic coatings to smart surfaces help avoid protein binding, keep important targets accessible and maintain control of surface charges after injection, has been confirmed by Sodipo and Mohammed (2024).Both the action of drugs and the safety of nanomaterials depend on their size, shape and surface charge. If nanoparticles are designed rationally for the different barriers in the body, it becomes easier to target tumors, they stay inside the tumor for longer and the body removes them less. If nanoparticles are improved, they are unlikely to fall apart early, will reduce harmful reactions outside the target area and won't tend to clump or form deposits in the blood. If particles are not properly developed, the immune system may see them as foreign substances, they may build up in the blood or stray to the liver, spleen or kidneys. For this reason, integrating chemical design with biological principles is very important for the success of nanomedicine in the clinic.

In the end, nanoparticles' biological process is mainly controlled by their size, shape and charge. Based on data in Figure 7 and research papers, macrophages take up particles within certain size and charge limits and so determine the clearance, interaction with the immune system and effectiveness of therapy. Appropriately and accurately working with these parameters helps produce safe, functional and efficient nanomedicine tools for diagnostics and therapy.

This chart explains why nanoparticle size, form and surface characteristics are important for uptake into cells. When compared, nanoparticles in the 30 nm–3 μm range with a spherical shape are better at being taken up and macrophage environments, where there are many phagocytes, seem to prefer highly negative or positive surface charges.

5. Mechanisms of Nanoparticle-Induced Oxidative Stress:

5.1. ROS Generation and Mitochondrial Dysfunction

Though mitochondria handle cellular energy metabolism and management of redox processes, they are key to creating and protecting cells from reactive oxygen species (ROS) in stressful situations. Interactions between engineered nanoparticles (NPs) and mitochondria have caught researchers' attention, as they are known to cause damage to the mitochondrial structure, lessen their respiratory activity and increase the amount of reactive oxygen species (ROS). Disruptions in mitochondrial movements—caused by activities of fission and fusion machinery—can explain many of the problems seen in the cell with nanoparticle exposure, as you can see in Figure 8.

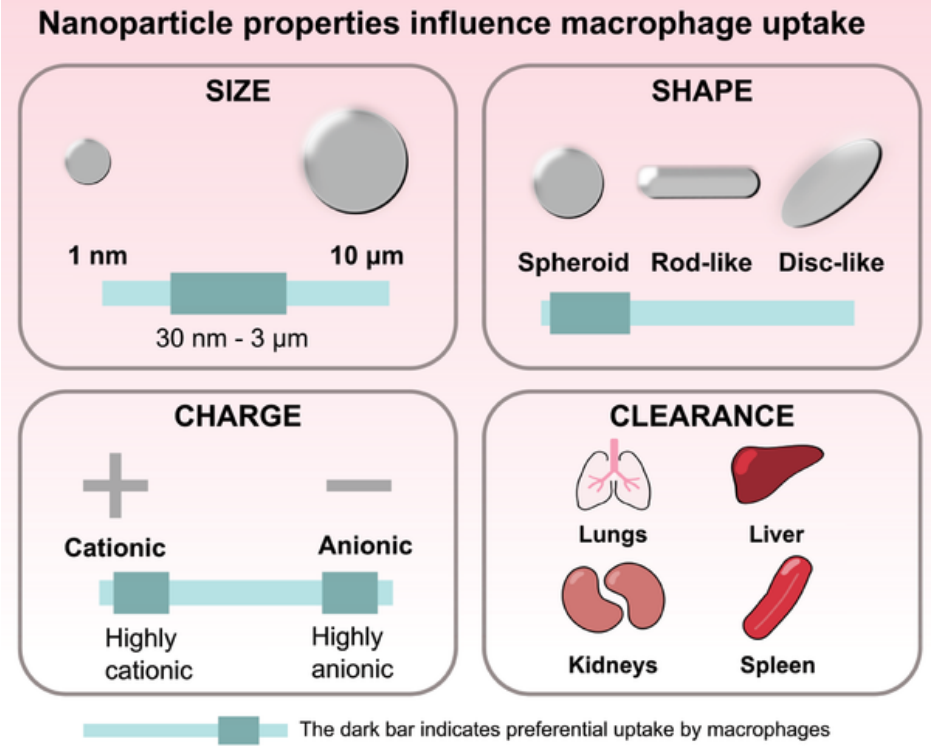


Figure 7: Impact of Nanoparticle Size, Shape, and Surface Charge on Cellular Uptake

Complexes I and III are where superoxide and other ROS are mainly formed after an electron discharge due to nanoparticle exposure in the electron transport chain (ETC). Silver (AgNPs), cobalt (CoNPs) and cerium oxide (nanoceria) disrupt mitochondrial function and increase ROS levels (Quan *et al.,* 2021; Chen *et al.,* 2023). In addition, nanoparticles that can take part in oxidation and reduction reactions such as iron oxide and those based on manganese, help produce highly reactive hydroxyl radicals through Fenton-like reactions. Nanoparticles interacting with the immune system cause the release of inflammatory cytokines which results in further ROS production mainly through the pathway of NOX4. Problems with autophagy and mitophagy cause an increase in damaged mitochondria, adding to the stress within the cell (Lv *et al.,* 2024).

The damaged mitochondria show various characteristics such as a collapse of the mitochondrial membrane potential (MMP), disrupted shape, ATP depletion and an initiation of apoptosis. Using JC-1 staining, studies confirm that cells exposed to NP have a major reduction in MMP which plays a crucial role in the initial failure of mitochondria (Shah & Dobrovolskaia, 2024). After NP treatment, there is usually a decrease in proteins that promote fusion such as OPA1 and mitofusins (MFN1/2) and an increase in proteins involved in fission such as Drp1 and FIS1, suggesting an imbalance toward more broken mitochondria (Qi *et al.,* 2020; Chen *et al.,* 2023). For this reason, oxidative phosphorylation is affected, cells lose energy and cytochrome c is released into the space between the outer mitochondrial membrane and other membrane—this initiates the intrinsic apoptotic program (Wang *et al.,* 2024).

New studies are considering using nanoparticles focused on mitochondria for research and treatment. Using triphenylphosphonium (TPP)-conjugated PLGA-curcumin nanoparticles has proven effective, as they reduce mitochondrial ROS and help keep cells healthy under stress (Gol & Gok, 2024). Diabetics then treated with

$BaTiO_3$-based piezoelectric nanoparticles showed suppressed oxidative stress in their mitochondria and an increase in mitophagy. Using nanoparticles with cerium and silica, the team managed to copy superoxide dismutase and catalase behavior, so they became helpful against ischemia in the brain (Nele *et al.,* 2023).

It has become clear in specific disease models that the interaction between NPs and mitochondria shapes the development of these conditions. Nanoceria treatment in cardiomyoblasts helped overcome Angiotensin II-induced hypertrophy by restoring mitochondrial functions and MMP (Gul *et al.,* 2023). By activating the NAD^+/SIRT1/PGC-1α pathway, these $Cu_{2-x}Se$ nanodevices helped increase mitochondrial biogenesis and performance in cases of Parkinson's disease (Zheng *et al.,* 2023). On the other hand, cobalt nanoparticles contribute to splitting the mitochondria and buildup of β-amyloid in C. elegans models of neurodegeneration which mitoquinone reverses (Chen *et al.,* 2023).

If nanoparticles are present, mitochondrial quality control is disrupted as a key consequence. If Drp1 and Mid51 are overproduced and MFN1, MFN2 and OPA1 are not present, mitochondria divide too much, grow small and stop functioning properly, not allowing them to heal. In a lot of cases, damaged mitochondria are not removed by mitophagy which intensifies oxidative stress (Lv *et al.,* 2024; Wang *et al.,* 2024). Other organelles, like the endoplasmic reticulum (ER), are also affected by the redox imbalance. Silver nanoparticles increased activity of NOX4 and the ER which caused more reactive oxygen species and therefore cell death by apoptosis in colorectal cancer cells (Quan *et al.,* 2021). In the opposite way, polyphenolic nanoparticles from pomegranate peel helped ease ER stress and supported the health of mitochondria in cardiomyocytes (Zheng *et al.,* 2024). Nanoparticles are being designed to inspect how mitochondria work. Targeting the ATP6 gene with ATP synthesis RNAi allowed the nanoparticles to significantly reduce ATP production and cause tumor cells to undergo immunogenic cell death, according to the authors of a new study (Xu *et al.,* 2023). Nanoparticles loaded with cisplatin and camptothecin that can target mitochondria have affected mitochondrial DNA, boosted the formation of reactive oxygen species and caused an increase in cancer cell apoptosis (Bajpai *et al.,* 2019) In short, nanoparticles work by making ROS and changing the function of mitochondria in both toxicity and medical uses. Out of control reactive oxygen species can damage organs, spark inflammation and lead to cell death, but targeted nanoparticle treatments can help fix issues in mitochondria and restore cellular balance. Based on Figure 8, the way mitochondrial fusion and fission occurs, the regulation of mitophagy and the creation of ROS play a main role in making nanomedicines safer and more efficient.

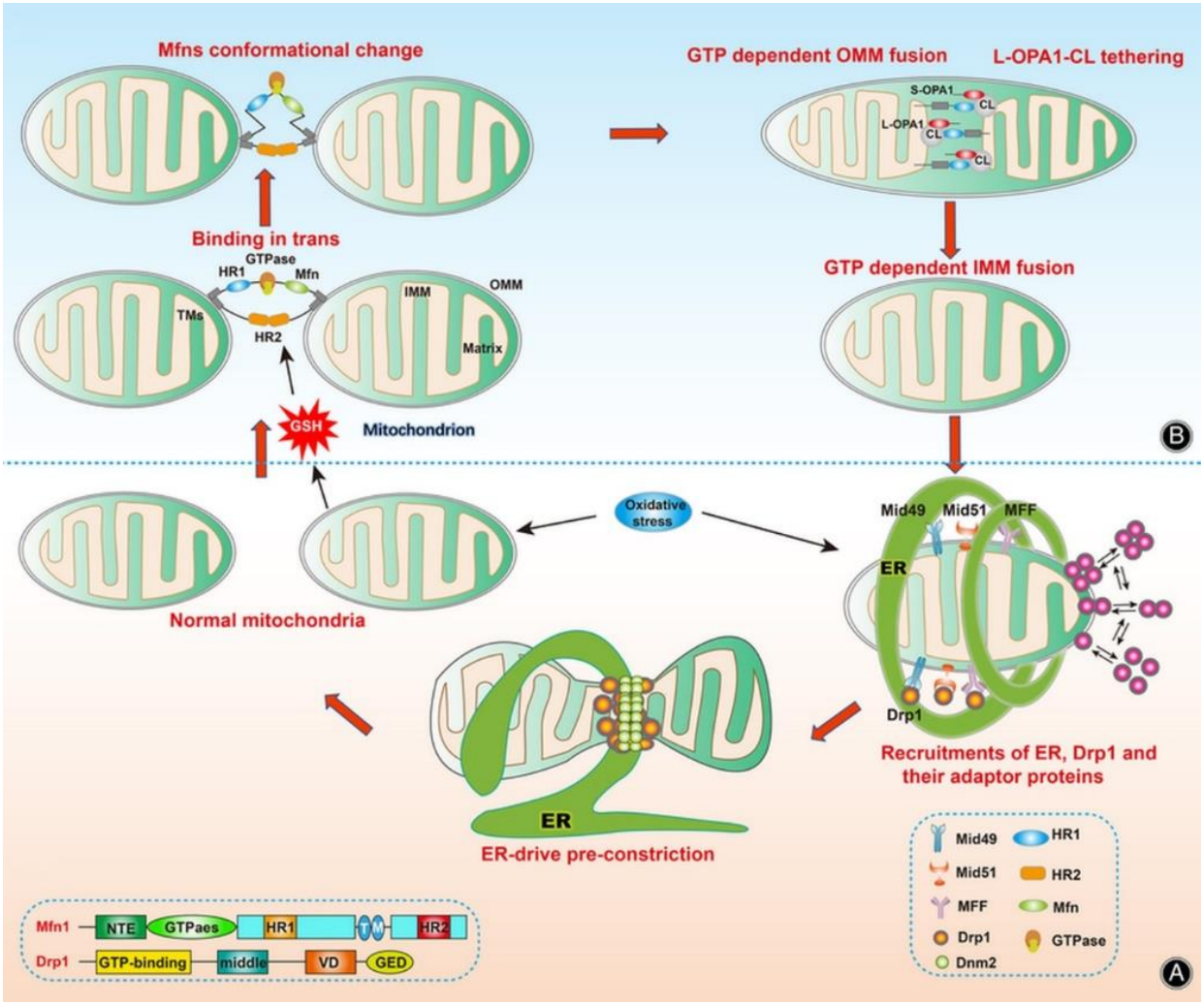


Figure 8: Nanoparticle-Induced ROS Generation and Mitochondrial Dysfunction

The diagram explains that oxidative stress influences the fusion and fission of mitochondria which regulates their shape. Whenever stress occurs, mitofusins (Mfns) and OPA1 help mitochondrial membranes fuse and Drp1 along with its adaptors (Mid49, Mid51, MFF) allows mitochondria to divide. When nanoparticles cause GSH to decrease and increase ROS, it disrupts the equilibrium which causes the mitochondria to break down, function improperly and induce apoptosis. The pre-constriction step driven by ER is crucial for getting fission machinery involved.

5.2. Disruption of Redox Homeostasis and Lipid Peroxidation

Micromanaging oxidation and antioxidant processes in cells is needed to prevent harmful changes to physiology. Most of the redox balance in the cell is looked after by key antioxidants, especially glutathione (GSH), catalase, superoxide dismutase (SOD) and glutathione peroxidase (GPX4). However, the unique chemical properties of nanoparticles (NPs), including their high surface area and reactivity, often interfere with this balance. After affecting cells, NPs can make too many reactive oxygen species (ROS), exhaust the cell's antioxidant protection and start lipid peroxidation as a sign of membrane injury and cell malfunction. Frequently, the first stage of disturbing redox balance comes from high ROS production which surpasses the ability of the body's endogenous antioxidants to control them. As an illustration, copper-gallic acid (Cu-GA) metal–phenolic network nanoparticles are known to lower cellular GSH, let Cu^{+} ions out to promote Fenton reactions and produce high amounts of ROS and an oxidation imbalance in this type of cancer cells (Zhao *et al.,* 2023). In a similar manner, Li et al. (2024) introduced nanoparticles that, once exposed to oxygen (redox reaction), became active in the presence of large amounts of GSH and aimed to release pro-oxidant agents that lead to a decrease in GSH, on top of promoting ferroptosis—which happens through lipid peroxidation and relies on iron. As a result of this study, the researchers found out that engineered nanoparticles can leverage the oxidative stress of cells to cause cell death.

Cellular antioxidants failing causes lipids to go through peroxidation. Chain reactions that damage cellular membranes are started by ROS, mainly hydroxyl radicals (•OH). Because of this, the integrity of the membrane is damaged, some signaling mechanisms are affected and frequently, the cell dies. Transition metal-based nanoparticles which could include iron or copper oxide NPs, cause more lipid damage by acting as catalysts for redox reactions that produce more ROS. Using Fe_3O_4@BSA-CE6 nanoplatforms, Fe^{2+} inside the cell triggered ferroptosis, causing more lipid peroxidation, when combined with photodynamic therapy to fight colorectal cancer (Zhang *et al.,* 2023). Besides causing ferroptosis, the buildup of lipid peroxides and linked aldehydes like malondialdehyde (MDA) and 4-HNE contributes to apoptosis and necrosis, making oxidative stress a major factor in cancer treatment and toxicity caused by nanomaterials.

Of all the factors involved in redox homeostasis, glutathione (GSH) stands out as the main antioxidant that both neutralizes ROS and assures cells keep their redox state. Low GSH levels are a major cause of ferroptosis which is a form of cell death involving an excess of iron and peroxidation of fats. Scientists have found that nanoparticles aimed at decreasing GSH are very effective at triggering ferroptosis. One example is that HSCPs (nanoparticles made with hollow copper peroxide) helped to keep ROS within cells and lessened GSH, preventing cancer stem cells in breast cancer models (by Xiong *et al.,* 2023). In a different work, Cao et al. (2024) produced nanoparticles that combined two agents: one that triggers ROS release and another that stops GSH synthesis in tumor cells. The double method of attack broken redox balance in cells and caused ferroptosis and apoptosis at the same time which made treating tumors more effective.

There are now various ways to increase the effects of oxidative stress by engineering the nanoparticles to avoid cellular detox systems. Those designed by Dey et al. (2024) use ascorbic acid and quinone methide, so when they release ROS they also decrease the amount of GSH inside the cell. Another sophisticated option is to use nanozyme-based systems. Ling and co-authors (2024) made hybrids between CuSe and AgNPs that show peroxidase activity and release ROS due to photothermal stimulation. In this way, they diminished glutathione and started oxidative damage which made the drugs more harmful to tumor cells. This shows that nanotherapy should focus on particles that produce ROS and also minimize the body's ability to defend against them so as to surpass the cell's stress barrier. Targeting cancer with these dual-action systems results in irreversible harms to cells and their eventual death. Exploiting oxidation reactions and lipid peroxidation is a central method to boost nanomedicine success and know more about nanoparticle toxicity.

5.3. Involvement of Inflammatory and Apoptotic Pathways

Making too many reactive oxygen species (ROS) upsets the balance in the cell and also activates inflammatory and cell death processes, mainly apoptosis. Cytokine levels increase, immune cells see more activity and there may be changes in how organs are structured in nanoparticle-caused inflammation which could result in either a benefit or damage depending on the level of exposure, the dosage used and the targeted organ. Changes in the immune system and oxidative stress form the main pattern in the effects engineered nanoparticles can have on the body. Often, the Toll-like receptors (TLRs) and nuclear factor-kappa B (NF-κB) activation are the first steps in triggering inflammatory signaling cascades. When these nanoparticles are present such as silver (AgNPs) and zinc oxide (ZnO NPs), they can cause the release of TNF-α, IL-1β and IL-6. How immunostimulatory the nanoparticles are can be controlled by changing their structure. A recent study revealed that genistein-loaded, ROS-sensitive nanoparticles lowered inflammation in inflammatory bowel disease (IBD) models by lowering the expression of caspase-1 and ASC, showing the usefulness of redox-reactive nanocarriers in managing this condition (Fan *et al.,* 2021). Just as environmental stressors can enhance the toxicity of nanoparticles, Wei *et al.,* (2024) found that marine heatwaves greatly enhanced the inflammatory response in mussels treated with TiO_2 nanoparticles

which happened through the alteration of the gut microbiota and excessive NF-κB activation. They stress the strong relationships between oxidative stress, immune reactions and various environmental factors.

Nanoparticles induce oxidative stress which often drives cells to die by apoptosis. The main parts of apoptosis are nuclear fragmentation, caspase activation and permeabilization of the mitochondrial outer membrane which are commonly caused by high ROS levels and issues in the mitochondria. When using doxorubicin-connected nanoparticles, inflammation occurring throughout the body in stroke and sepsis is reduced by selectively causing neutrophils to die (Zhang *et al.,* 2019). Frequently, cytochrome c is released from broken mitochondria after exposure to nanoparticles which triggers the intrinsic caspase pathway and provokes apoptosis.

Studies done recently suggest that both apoptosis and ferroptosis, a cell death that depends on iron-related lipid peroxidation, are connected. When ferroptosis and apoptosis are combined in a therapy, they often work together in a synergistic way. Huang *et al.,* (2024) created liposomes designed to cause ferroptosis, apoptosis and photodynamic therapy, resulting in a more effective killing of cancer cells by combining the different cell death processes. Similarly, Ren et al. (2021) built nanoparticles that target the mitochondria of cancer cells which causes a loss of glutathione and GPX4 and causes cell death in two ways, ferroptosis and apoptosis, plus immunogenic cell death. On top of causing apoptosis, nanoparticles have also been proved to alter the whole immune system. The impact of nanoparticle-induced inflammation on the immune system can be either harmful (immunosuppression) or boosting (immunostimulation), depending on the circumstances. When inflammation is excessive, it may harm lymphocytes and lower the immune system, but moderate ICD helps release special molecules that signal and boost adaptive immunity. It is very helpful for fighting cancer. Research by Cao *et al.,* (2024) found that erastin (to induce ferroptosis), FdUMP (a chemotherapeutic) and siPD-L1 (to block PD-L1) combined via lipid nanoparticles simultaneously triggered apoptosis, ferroptosis and the immune system in colon cancer cells.

Both local and general health problems may happen because of exposure to nanoparticles. It is known that cardiovascular toxicity is connected to cell death (apoptosis) because of oxidative stress as well as inflammation in blood vessels (endothelial tissue). The authors in Clinova *et al.,* (2024) discussed the adverse effects of engineered nanoparticles on the heart, pointing out that they generally result in endothelial injury, increased leaking from blood vessels and cardiac problems due to the release of free radicals and inflammation. In acute lung injury experiments, nanoparticles were found to harm mitochondria, mainly by disrupting the DRP1/MFN1 system which led to cytochrome c release and caused both inflammation and scarring in the lung (Yan *et al.,* 2025). In short, the damage of redox balance by nanoparticles, together with the effects of lipid peroxidation, results in inflammation and apoptosis in the body. They both increase the risk of side effects in areas we don't mean to affect and also open opportunities for targeted therapy. Improvements in nanoparticle technology have made it possible to direct immune system responses for fighting cancer. But professional monitoring and tweaking prescribed drugs is important to avoid problems and ensure treatment is successful with the least risks.

6. Enzymatic Biomarkers of Oxidative Stress:

6.1. Superoxide Dismutase (SOD), Catalase (CAT), and Glutathione Peroxidase (GPx)

When oxidative stress is caused by engineered nanoparticles, three principal antioxidant enzymes manage the body's response: superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx). These enzymes are the main defenses within cells against reactive oxygen species (ROS), mainly in places that are either very active or encounter external stressors including NPs. The $O_2^{\bullet -}$ radical is turned into hydrogen peroxide (H_2O_2) by SOD which is then broken down into water and oxygen by CAT mainly inside peroxisomes. GPx is especially active in the mitochondria and cytosol, helping to reduce H_2O_2 and lipid hydroperoxides by breaking them down with glutathione (GSH). Enzymes play a role in maintaining redox balance, so disturbances in their function after nanoparticle exposure set off oxidative stress and make lipids, proteins and DNA in cells more vulnerable to injury.

A lot of studies have indicated that some nanoparticles can prevent these enzymes from working which causes oxidative damage. In just four days of swallowing titanium dioxide nanoparticles (TiO_2-NPs), Nile tilapia experienced significant decreases in SOD (−27%), CAT (−60%) and GPx (−37%). As the study found, continuous use of nanoparticles for over fourteen days caused the upregulation of enzymes which hints at a protective feedback system (Fırat & Bozat, 2019). AgNPs in HaCaT keratinocytes reduced the mRNA expression of SOD1, CAT and GPX-1 and this effect depended on the concentration of AgNPs used, so it seemed that AgNPs impaired the antioxidant defense system at the genetic level (Habas & Shang, 2019). Still, several researchers have found that nanoparticles can help improve the antioxidant response of the body or duplicate certain enzyme functions. Giving Au/Ag/Fe nanoparticles to cells with colon adenocarcinoma brought back SOD, CAT and GPx functions and lowered the amount of MDA and diene conjugates (Andriychuk *et al.,* 2023). In lung cells treated with hydrogen peroxide, platinum nanoparticles (PtNPs) produced the same results, boosting enzymatic functions and decreasing damaged DNA (Ismail *et al.,* 2022).

Because of nanozymes—nanodimensional substances that act like typical enzymes—oxidative stress can now be kept in check in more ways. The Zhang group showed that the use of MnO_2-BSA nanoparticles in BEAS-2B lung cells allowed them to function similarly to SOD, CAT and GPx which lowered H_2O_2 amounts and minimized apoptotic processes by regulating Bax and Bcl-2 (Zhang *et al.,* 2022). Ye et al. found that calcium hexacyanoferrate nanoparticles (CaHF NPs) can reduce ROS, stimulate enzymatic activities and control inflammatory cytokines in hypertensive rats (Ye *et al.,* 2023). $MoSe_2$-PVP nanoparticles were able to preserve pancreatic tissue in acute pancreatitis models by decreasing oxidative injury by playing a similar role as SOD, CAT and GPx (Xie *et al.,* 2022).

Antioxidant enzymes respond differently to NPs in different organs. In both the brain and the liver, silica nanoparticles modified with ascorbic acid (SiO_2-NPs@AA) helped to recover from H_2O_2 toxicity by increasing the activities of SOD, CAT and GPx in the cortex and hippocampus (Hamdi *et al.,* 2025). By contrast, the activity of antioxidant enzymes was suppressed in the gills and liver of Oreochromis niloticus after 3 to 7 days of exposure to aluminum oxide nanoparticles, signifying a fast and uncompensated impact on the fish (Temiz & Kargın, 2021). How energy enzymes express their functions can be also controlled by nanoparticle properties such as the type of material, size and charge and by environmental pressures. By presenting SOD and CAT on Au/Ag nanoparticles and then using them on UV-exposed rat skin, the team found that their activity was increased and that oxidative stress on DNA was decreased (Pudlarz *et al.,* 2020).

Comparing different species has offered extra understanding about their antioxidant systems. After exposure to copper oxide nanoparticles (CuO NPs), Galleria mellonella larvae showed higher CAT activity and lower SOD activity, illustrating how copper impacts these two different tissues in distinct ways (Tuncsoy *et al.,* 2019). Likewise, AgNPs and ionic Ag^+ similarly increased levels of SOD, CAT and GPx in the earthworm Aporrectodea caliginosa and ionic silver had more of an effect because it was more soluble and readily available to organisms (Saleeb *et al.,* 2020). In the case of marine mussels (Mytilus galloprovincialis), exposure to nickel oxide nanoparticles (NiO-NPs) increased the SOD activity, but in general, the CAT and GPx enzymes behaved differently and indicate that aquatic organisms have different thresholds for sensitivity to enzymes (Gürkan, 2022).Taken together, SOD, CAT and GPx function as both detectors of oxidative damage triggered by nanoparticles and targets for treatment with nanoparticles. Rather than depleting the defense system, some engineered nanoparticles support and grow antioxidant defenses to repair ROS oxidative damage. Understanding the ways enzymes react with nanoparticles in various tissues and organisms is important for improving nanomaterial safety and their medical usages.

6.2. Enzyme Kinetics and Activity Assays in Exposed Cells

Analyzing enzymatic activity and kinetics of cell defense enzymes superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx) after exposing cells to NPs reveals the molecular events that cause oxidative stress. Studying enzyme kinetics allows researchers to compute reaction speed, enzyme–substrate interaction and catalytic conversion, helping them understand biological changes, see what levels of toxicity may emerge and test protection against nanoparticles. Analysts closely monitor Vmax (maximum velocity), Km (the substrate concentration needed for half-maximum activity) and kcat (turnover number) to judge whether enzymatic changes stem from induction, inhibition or compensation. Changes in their levels after exposure to nanoparticles assist in telling adaptive cell protection from nanoparticle toxicity.

Standard tests on the activities of certain enzymes are the foundation of oxidative stress assays in studies involving nanoparticles. Nitroblue tetrazolium (NBT) reduction inhibition and the pyrogallol autoxidation method are common ways to evaluate SOD activity. CAT is measured by checking the speed at which hydrogen peroxide (H_2O_2) is decomposed at 240 nm, while GR-coupled assays that use NADPH and can be observed at 340 nm are typically how GPx activity is measured. Different cell and tissue lines use these assays to determine how much the antioxidant capacity is influenced by nanoparticles.

In experimental conditions, enzyme activity tends to first increase at low doses of nanoparticles as a protective stress reaction, but then decrease at high doses due to either high levels of ROS they produce or direct harm to the enzymes. Research in 2020 found that prolonged contact of the African catfish with PVC microplastics lowered both SOD and CAT activity in their brain and gills, suggesting that such sustained contact can reduce their ability to deal with oxidative stress (Iheanacho & Odo, 2020). In contrast, an increase in SOD and CAT gene expression was observed in HT-29 colon cells caused by exposure to silver, silica and zinc oxide nanoparticles over time which may suggest that cells are doing this to balance the ROS accumulation (Budak, 2019).

Responses of enzymes in different tissues to exposure to nanoparticles have also been observed. In zebrafish embryos receiving silver nanoparticles, higher levels of SOD and CAT were noticed in their livers and the other tissues (such as muscle and brain) were not changed (Amiri *et al.,* 2023). Likewise, in cultured plant cells, aluminum-based nanoparticles tended to activate glutathione reductase (GR) instead of CAT or SOD which might indicate that the choice of antioxidant

system depends on both tissue and the type of nanoparticle (Ameri *et al.,* 2020).

Enhancements in nanozyme research now allow for the study of enzymes that are found in systems with nanoparticles. Nanozymes, small particles made to act like enzymes, are able to remain stable and active in various conditions inside the body. Gold-silver-apoferritin nanozymes act like SOD, CAT and GPx and their reported catalytic constants are kcat=1.4×10^6 s^{-1} for SOD, 0.1 s^{-1} for CAT and 9×10^3 s^{-1} for GPx (Dashtestani *et al.,* 2019). Comparably, enzyme-like activities can be restored in BEAS-2B cells suffering from oxidative stress using MnO_2-BSA nanoparticles which keep removing H_2O_2 and are not easily broken down, making them great candidates for redox-controlled therapeutics (Zhang *et al.,* 2022). The comparison between in vitro and in vivo models has brought to attention further issues in explaining the role of enzymes after NP exposure. Because there is direct contact between cultured cells and nanoparticles, in vitro models quickly experience notable enzyme activity increases. In living systems, metabolism, differences in where drugs go and communication between organs often reduce or delay the effects expected. Exposing rats with colon cancer to Au/Ag/Fe nanoparticles made the levels of SOD, CAT and GPX liver enzymes normal, whereas in isolated hepatocytes there was only a short-term increase that was very sensitive to dose (Andriychuk *et al.,* 2023).

Yet, there are a number of constraints involving the usage of enzymatic tests in the field of NP toxicology. Some metal oxide nanoparticles may interfere with spectrophotometric assays because they absorb or scatter light at wavelengths where the measurement is made. Apart from that, nanoparticles could adsorb enzymes by accident which might lower their results in the assay. Sometimes using atypical pH or ion concentrations in the test buffer does not truly reflect how an enzyme works in the body. This calls for designing experiments carefully, with control groups that have no nanoparticles and verification of interference from light.

Besides studying enzymes, looking at nanoparticle impacts on enzyme kinetics can be useful in disease prevention and patient care. Looking at the activity of antioxidant enzymes as they respond to a dose can be used to track the beginning stages of cell poisoning. Recovery of enzyme function after the treatment is done can prove that the treatment provided was successful. Also, by using biosensors that have enzymatic components, scientists have been able to make advances in finding ROS. Combining SOD or GPx with electrochemical sensors has been proved useful for real-time cancer screening and measurements of oxidative stress, as the sensors are fast and accurate (Vincent *et al.,* 2021). Basically, using enzyme kinetics and activity assays allows researchers to understand how nanoparticles work and what benefits they bring to biological systems. Even though conventional assays help explain cell reaction to substances, biosensing and nanozymes are changing the way we study and manage the effects of nanoparticles on cells in medicine.

6.3. Dose- and Time-Dependent Enzymatic Response to Nanoparticles:

Different doses and exposure times of nanoparticles (NPs) cause a variety of oxidative effects, while the behavior of superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx) is dynamic as a result. They guard the body against reactive oxygen species (ROS) and their action depends on the amount as well as the length of exposure. Often, when the dose of NP is not too high, the body increases its enzyme activity as a way to protect itself. If exposure lasts for a long time or happens in high doses, the system may get overloaded and the enzymes can't work properly because of the excess ROS or enzyme damage. Prolonged contact with the toxin might set up a defense response in some cells or it can permanently damage them based on the cell or tissue. The way a drug response happens to nanoparticles depends a lot on their size, shape, surface charge and the elements they contain.

There is strong evidence from experiments that dose-related changes happen. As an example, a concentration of 1 mg/L aluminum oxide NPs in the water increased the CAT and GPx levels in Nile tilapia after 3 days, but this was reversed as the concentration increased to 5 or 10 mg/L by day 7 and enzyme activity was decreased (Temiz & Kargın, 2021). For rats with colon cancer who received gold/silver/iron nanoparticles at moderate doses (0.1–0.2 mg/kg), antioxidant enzymes SOD, CAT and GPx went back to normal, but large doses caused oxidative stress and decreased the activity of inflammatory enzymes (Andriychuk *et al.,* 2023). In another instance, exposure of earthworms (Aporrectodea caliginosa) to silver nanoparticles caused the activity of SOD and CAT to rise up to 72 hours, but high concentrations of the nanoparticles (10 mg/kg) decreased their levels because of oxidative stress and glutathione loss (Saleeb *et al.,* 2020). The same change occurred in plant roots exposed to zinc oxide NPs, with initial elevation of GPx and SOD decreasing later, thought to be because of enzyme damage or interaction with the toxic nanoparticle surfaces (Jahantab *et al.,* 2022).

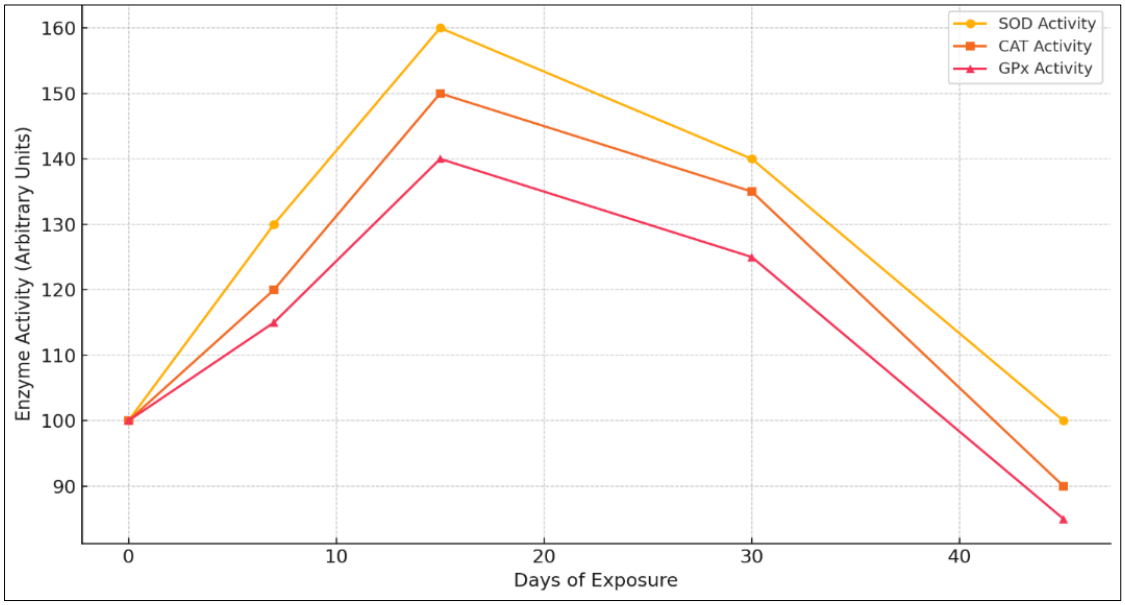
How and when the child is exposed is very important as well. A study that lasted 45 days on African catfish found that after exposure to polyvinyl chloride microparticles on day 30, CAT and SOD activity decreased, showing that the enzymes were being used up (Iheanacho & Odo, 2020). When treated with nano-titanium dioxide, the livers of mice had high levels of enzymes for 48 hours, but later on, enzymes and signs of mitochondria dysfunction and tissue damage dropped by 72 hours (Chen *et al.,* 2021). In cell culture experiments with HT-29 colon cells, after ZnO NP exposure, SOD and CAT gene expression rose fast in the first 6–12

hours, only to reduce over the next 24 hours, symptoms of a brief antioxidant defense strategy (Budak, 2019).

Species and tissue differences in how enzymes work make the problem more complicated. In Mytilus galloprovincialis, nickel oxide NPs caused an increase in SOD and CAT activity in gill tissue, but there was no major change found in digestive gland cells days later (Gürkan, 2022). When treated with calcium hexacyanoferrate nanozymes, Arabidopsis and tomato showed marked improvement in SOD (46%), CAT (48%) and GPx (74%) by day 7 and the changes were still noticeable up to day 14 (Shen *et al.,* 2024).

Interestingly such enzymatic models are observed in both toxicological situations and in diseases. Those people living with schizophrenia for a greater period and who experienced many psychosis episodes were found to have a lower GPx activity, suggesting that continuous oxidative stress may drive the disease regardless of environment exposure (Djordjević *et al.,* 2022). SOD and CAT showed increased activity in diabetic ulcer tissues at the beginning of inflammation, but the ongoing oxidative injury and degeneration caused a decrease (Bhattacharyya, 2019). They show that enzyme function can be affected by strong oxidative stress occurring at different times, even in real medical situations.

Nanozymes which act like enzymes, are able to perform strongly and stably over different time and dose exposures to oxidants. $MoSe_2$-PVP nanozymes were able to steadily carry out SOD and GPx activities even during inflammatory conditions for more than 48 hours in pancreatitis models which is superior to native enzymes maintaining redox balance (Xie *et al.,* 2022). MnO_2-BSA nanozymes also showed the highest resistance to inactivation by oxidative stress, getting rid of hydrogen peroxide in the body for up to 72 hours (Zhang *et al.,* 2022). For this reason, nanozymes are expected to play a bigger role in dealing with ongoing or repeated times of oxidative stress, where ordinary enzymes cannot be trusted for their instability or fast use-up. All of these happen together, so that the response of enzymes to nanoparticles depends on their amount, how long they are present, the tissue they affect and the chemical make-up of the nanoparticles. Thorough study of these results helps create better models for predicting nanotoxicity and engineering better uses of nanoparticles and nanozymes in fighting oxidative stress.



Graph1: Time-Dependent Antioxidant Enzyme Response to Nanoparticle Exposure

7. Genetic and Epigenetic Responses to Oxidative Stress:

7.1 Gene Expression Alterations in Antioxidant Defense Pathways:

A frequent result of NPs is an overproduction of reactive oxygen species (ROS) that causes oxidative stress and either switches on or off genes that help protect the body's cells. The defense response relies heavily on the Nrf2-Keap1 signaling pathway which in turn controls genes for antioxidant enzymes SOD, CAT and GPx. Once Keap1 stops blocking it during stress, Nrf2 travels to the nucleus and activates the genes that contain the antioxidant response element (ARE). A number of studies suggest that the size, dose and duration of exposure to metal-based NPs like ZnO and AgNPs can cause them to either improve or weaken the cellular response to oxidative stress regulated by Nrf2 (Thiruvengadam *et al.,* 2023).

Wild and epithelium cells that were exposed to silica nanoparticles (SiNPs) showed changes in HO-1, NQO1 and GST genes, suggesting that the cells had

switched on cytoprotective defense (Zheng *et al.,* 2024). Before cytotoxic changes, these alterations might warn that cells are reacting to oxidative stres Green-synthesized nanoparticles are able to change gene activity in more compatible ways with biology. For example, Khatik (2021) found that green nanomaterials can activate antioxidant and anti-apoptosis genes and, by doing so, reduce gene activation related to oxidative stress which could make them useful for medical care.

7.2 DNA Damage, Repair Mechanisms, and Genomic Instability:

Oxidative stress mainly aims to attack DNA. Specific types of nanoparticles which can trigger ROS either by Fenton effects or by disrupting the mitochondria, are able to cause single-strand breaks, double-strand breaks and lead to the formation of 8-oxoGuanine lesions. Without repair, these lesions can lead to changes in the DNA, breaks in chromosomes and also cancer.

Such damage prompts the start of base excision repair (BER), nucleotide excision repair (NER) and mismatch repair (MMR), all of which are activated swiftly. Nevertheless, too much exposure to NPs is known to either overburden or suppress these body systems. Bonadio et al. (2020)'s study found that maghemite nanoparticles brought about damage to DNA and modifications in the amounts of XRCC1 and OGG1 repair genes in human glandular cells.

The combination of DNA repair with epigenetics generates new types of challenges. A common impact of NPs is to silence DNA repair genes by methylating their promoters which makes it difficult for cells to react to genotoxic damage (Ghosh *et al.,* 2022). A loss of genomic stability is a key feature of cancer, aging and degenerative diseases.

For this reason, new evidence points out that nanoparticles such as silica and titanium increase risks by weakening genome stability, shortening telomeres and affecting the expression of important checkpoint genes p53, ATM and ATR (Zheng *et al.,* 2024; Fragou & Kovatsi, 2021). Depending on DNA damage and the ability to repair it such changes can result in cell cycle shutdown or apoptosis.

7.3 Epigenetic Modifications: Methylation, Histone Changes, and miRNA Regulation:

Tiny nanoparticles also interact with the epigenome, bringing about gene expression changes that are handed down across generations and can be changed. Some changes you might try are:

Risks for altered DNA methylation can come from NP exposure as it leads to oxidative stress. There are studies showing that methylation of promoters involved in inflammation and apoptosis is disrupted by drug nanoparticles (Brzóska *et al.,* 2019). In numerous circumstances, dysfunctional methylation of DNA can cause genes to either stop working or start working which can harm or kill cells. In one example, nanoparticles synthesized by marine bacteria affected the methylation of DNMT genes and many CpG islands, changing how accessible the genes were (Patil *et al.,* 2019). NPs help determine histone acetylation and methylation which manage chromatin structure and the openness of genes. When used in bladder cancer, nZnO was seen to lower H3K27me3 on the RUNX3 gene which then led to a rise in its expression and more apoptosis (Zhang *et al.,* 2020). Because of these histone marks, the chromatin can stay flexible to allow for transcription factors which are important for genes to function.

MicroRNAs (miRNAs) are small RNAs that are important for controlling genes after they are made. It has been found that NP exposure can cause major disruption in the expression of miRNAs. Their findings showed that metal nanoparticles in HepG2 cells caused changes in miR-34a, miR-21 and miR-155 expression (Brzóska et al. 2019). They are linked to the regulation of both inflammations, the rapid occurrence of cells and programmed cell death. Raising or lowering the amount of these regulatory elements could help us understand some of the harm NPs do to cells.In addition, treatments that use miRNA in nanocarriers are promising. A study shows that miR-217-5p in polymeric nanoparticles reduced the growth of glioblastoma stem cells by stopping the activity of EZH2, a histone methyltransferase (Korleski *et al.,* 2024). This points out that nanoparticles can regulate as well as supply epigenetic therapies.

Together, nanoparticles can lead to a shift in how cells respond to stress caused by free radicals via many genetic and epigenetic mechanisms. Modifications in gene expression, DNA and its repair and changes in DNA methylation, histones and miRNA, cause the different biological results observed in nanotoxicology. It is important to understand these effects to judge whether nanoparticles are safe and how to benefit from them in precision medicine.

8. Advanced Analytical and Molecular Techniques:

8.1 Detection of ROS and Oxidative Stress Biomarkers

The accurate detection of reactive oxygen species (ROS) and their associated oxidative stress biomarkers is pivotal for elucidating the molecular mechanisms underlying a wide range of pathological conditions, including cancer, diabetes, and neurodegenerative diseases. ROS are naturally generated as byproducts of mitochondrial respiration and cellular metabolism; however, their excessive accumulation disrupts redox homeostasis, leading to oxidative damage to cellular macromolecules such as lipids, proteins, and nucleic acids (Umeno *et al.,* 2017).

Modern analytical techniques have greatly enhanced the precision and reliability of oxidative stress detection. Commonly measured biomarkers include lipid peroxidation products such as malondialdehyde (MDA) and isoprostanes, along with DNA oxidation indicators like 8-hydroxy-2′-deoxyguanosine (8-OHdG). Sophisticated methods such as liquid chromatography-tandem mass spectrometry (LC-MS/MS), flow cytometry, and fluorescence-based imaging probes now enable dynamic and in situ detection of these oxidative markers, supporting both clinical diagnostics and environmental health assessments (Fanti *et al.,* 2025; Li *et al.,* 2023).

Emerging non-invasive diagnostic approaches, such as the analysis of exhaled breath condensate (EBC), offer promising alternatives for detecting volatile and soluble oxidative stress markers like hydrogen peroxide and F2-isoprostanes. These techniques enhance patient compliance and expand the scope of oxidative stress evaluation in population-based studies (Rahman & Biswas, 2004). Despite these advances, several limitations persist. The lack of standardization in analytical protocols across laboratories, as well as the variable specificity of some oxidative biomarkers in multifactorial disease contexts, continues to hinder their broader clinical application (Ruskovska & Jansen, 2012). Additionally, biological variability and the transient nature of ROS further complicate quantitative assessments.

In conclusion, while cutting-edge technologies have significantly improved the detection of ROS and oxidative stress biomarkers, further validation, standardization, and integration into clinical workflows are essential to realize their full diagnostic and prognostic potential.

8.2 qRT-PCR, Microarrays, and RNA-seq for Gene Expression Profiling:

Gene expression profiling plays a critical role in elucidating cellular responses to oxidative stress, understanding disease mechanisms, and evaluating therapeutic interventions. Three principal techniques—quantitative real-time PCR (qRT-PCR), microarrays, and RNA sequencing (RNA-seq)—are widely employed in transcriptomic analysis, each with specific advantages and constraints. qRT-PCR is recognized as the gold standard for the precise and sensitive quantification of selected gene transcripts, making it particularly valuable for validating expression patterns identified in broader screens. However, its limited throughput restricts its application in large-scale studies (Sun *et al.,* 2012). In contrast, microarrays enable the simultaneous measurement of thousands of genes and have been extensively used in oxidative stress research due to their cost-effectiveness and reproducibility. Nonetheless, they are less sensitive to low-abundance transcripts and novel sequences, often underperforming in detecting subtle changes when compared to RNA-seq and qRT-PCR (Noel *et al.,* 2014). RNA-seq has emerged as the most comprehensive tool for transcriptomic profiling, offering superior sensitivity, broader dynamic range, and the ability to detect novel transcripts, splice variants, and non-coding RNAs. It consistently identifies a greater number of differentially expressed genes and shows stronger concordance with qRT-PCR validation than microarrays (Lahiry *et al.,* 2011). Studies have demonstrated that RNA-seq can uncover nuanced gene expression shifts under oxidative stress conditions that are undetectable by traditional microarrays, providing deeper insight into regulatory mechanisms (Liu *et al.,* 2013). Furthermore, integrating these methodologies enhances the robustness of gene expression analysis; for example, RNA-seq and microarrays have been shown to identify overlapping yet distinct gene sets, highlighting their complementary roles in capturing the complexity of oxidative stress-induced transcriptomic changes (Kogenaru *et al.,* 2012).

8.3 Proteomics, Western Blotting, and Enzyme-Linked Assays:

Proteomic analysis, Western blotting, and enzyme-linked assays are essential tools for investigating oxidative stress at the protein level, providing insights into altered protein expression, post-translational modifications, and cellular signaling pathways. Proteomics, particularly mass spectrometry-based approaches, enables the large-scale identification and quantification of proteins affected by oxidative stress, including oxidatively modified proteins, stress-responsive enzymes, and redox-regulated signaling molecules. Advanced proteomic platforms allow for the detection of carbonylation, nitrosylation, and other oxidative modifications, facilitating a deeper understanding of the molecular consequences of redox imbalance (Butterfield *et al.,* 2014). Western blotting remains a cornerstone technique for validating proteomic findings and analyzing the expression of specific proteins involved in oxidative stress responses, such as superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx). This technique offers high specificity and semi-quantitative assessment of protein levels and modifications. Meanwhile, enzyme-linked immunosorbent assays (ELISAs) provide a sensitive, high-throughput means for quantifying target proteins and oxidative stress biomarkers, including 8-hydroxy-2′-deoxyguanosine and protein carbonyls, in biological fluids and tissues (Dalle-Donne *et al.,* 2003). Together, these methods form a complementary toolkit for assessing protein-level changes under oxidative stress conditions, with proteomics offering a broad systems-level view, Western blotting providing targeted validation, and ELISA enabling scalable quantification across diverse sample types. The integration of these techniques is increasingly employed in clinical and experimental settings to elucidate redox biology and its implications for disease progression and therapeutic interventions.

9. Implications for Nanomedicine and Safety Evaluation

9.1 Risk Assessment and Regulatory Considerations

Risk assessment in nanomedicine requires a nuanced understanding of the unique physicochemical properties of nanoparticles, which influence their toxicity, biodistribution, and persistence in biological systems. Unlike conventional substances, nanoparticles possess enhanced surface reactivity and the ability to penetrate biological barriers such as cell membranes and the blood–brain barrier. These capabilities raise concerns about bioaccumulation and long-term systemic effects (Fadeel *et al.,* 2018). Traditional toxicological approaches often prove inadequate for assessing these risks, necessitating the development of specialized frameworks. Regulatory agencies, including the U.S. Food and Drug Administration (FDA) and the European Medicines Agency (EMA), have stressed the importance of case-specific evaluations and are progressively integrating nano-specific criteria into their regulatory processes. Critical parameters such as particle size, surface area, surface charge, and solubility are now being recognized as essential for toxicity evaluation (Krug, 2014). The European Union's REACH (Registration, Evaluation, Authorisation, and Restriction of Chemicals) regulation has also incorporated provisions specifically for nanoforms, mandating tailored registration and safety data requirements. However, a major obstacle in the regulatory landscape remains the lack of standardized protocols for nanoparticle characterization and toxicity testing, which hampers data comparability and international regulatory harmonization (Nel *et al.,* 2013).

9.2 Strategies for Safer Nanoparticle Design

The design of safer nanoparticles is an active area of research focused on minimizing toxicity while preserving or enhancing therapeutic functionality. One core strategy involves using biocompatible and biodegradable materials, such as poly(lactic-co-glycolic acid) (PLGA) and liposomes, to reduce systemic toxicity and facilitate safe degradation. Surface functionalization techniques, including PEGylation (polyethylene glycol coating), are commonly employed to prevent rapid clearance by the immune system and to extend circulation time (Poon *et al.,* 2020). The emerging "safe-by-design" approach integrates toxicological screening early in the development pipeline, allowing researchers to iteratively modify nanoparticle formulations based on safety profiles without compromising performance (Giannakou *et al.,* 2020). This methodology not only reduces the risk of late-stage failure but also aligns with regulatory expectations for proactive risk management. Furthermore, the use of predictive in silico models and high-throughput screening platforms is enhancing the ability to preemptively identify hazardous physicochemical traits, thereby streamlining the development of safer nanomaterials (Halappanavar *et al.,* 2019).

9.3 Translational Potential in Drug Delivery and Diagnostics

Nanoparticles offer transformative potential in the fields of drug delivery and diagnostics due to their ability to enhance solubility, stability, and targeted delivery of therapeutic agents. Clinically approved formulations, such as liposomal doxorubicin (Doxil) and lipid nanoparticles used in COVID-19 mRNA vaccines, exemplify successful translation from bench to bedside by leveraging nanoparticle-based systems to improve pharmacokinetics and bioavailability (Hou *et al.,* 2021). Additionally, theranostic nanoparticles, which combine diagnostic and therapeutic functionalities in a single platform, have gained traction in precision medicine—particularly for cancer and infectious diseases—by enabling real-time monitoring and individualized treatment strategies (Wang *et al.,* 2021). Despite these advancements, significant challenges remain in achieving consistent large-scale manufacturing, maintaining batch-to-batch reproducibility, and mitigating immunogenic responses. Addressing these barriers requires interdisciplinary collaboration among material scientists, toxicologists, clinicians, and regulatory stakeholders to ensure that innovative nanotechnologies are translated safely and efficiently into clinical practice.

10. CONCLUSION AND FUTURE PERSPECTIVES

10.1 Summary of Key Findings and Mechanistic Insights

This review highlights the complex interplay between nanoparticles and biological systems, emphasizing the pivotal role of oxidative stress in mediating nanotoxicity. Mechanistic insights gathered from in vitro and in vivo studies reveal that the physicochemical properties of nanoparticles—such as size, surface area, charge, solubility, and shape—critically influence cellular uptake, biodistribution, and toxicity profiles (Fadeel *et al.,* 2018; Krug, 2014). Notably, oxidative stress emerges as a central mechanism, leading to lipid peroxidation, DNA damage, mitochondrial dysfunction, and impaired antioxidant defenses (Nel *et al.,* 2013; Halappanavar *et al.,* 2019). Enzymatic biomarkers, such as superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx), consistently demonstrate perturbations upon nanoparticle exposure, suggesting their potential utility in nanotoxicological assessment (Hou *et al.,* 2021). Moreover, advances in transcriptomic and proteomic profiling have shed light on nanoparticle-induced gene expression disturbances and cellular signaling disruptions, contributing to a more nuanced understanding of nanoparticle–cell interactions (Poon *et al.,* 2020).

10.2 Current Gaps and Challenges in the Field

Despite considerable progress, critical challenges persist in the field of nanotoxicology. One of the foremost issues is the lack of standardized testing

protocols, which limits cross-study comparability and regulatory alignment (Krug, 2014). The heterogeneity in nanoparticle synthesis, surface modification, and dispersion stability further complicates toxicity prediction and reproducibility (Giannakou *et al.,* 2020). Additionally, long-term and chronic exposure studies remain scarce, hindering accurate assessment of bioaccumulation, biopersistence, and delayed toxic effects (Fadeel *et al.,* 2018). The insufficient integration of high-throughput screening, multi-omics data, and computational modeling in routine safety evaluations restricts our ability to develop predictive frameworks. Furthermore, regulatory frameworks, although evolving, often lag behind technological innovation, creating uncertainty for stakeholders in biomedical applications (Nel *et al.,* 2013; Wang *et al.,* 2021).

10.3 Emerging Directions for Safer Nanotechnology in Human Health

Future research must adopt an interdisciplinary, proactive approach to address current limitations and guide the development of safer nanotechnologies. The "safe-by-design" concept, which emphasizes early toxicological screening and iterative design modifications, is gaining momentum as a strategy to mitigate nanoparticle-associated risks (Giannakou *et al.,* 2020). Predictive in silico modeling, machine learning algorithms, and systems biology approaches are increasingly being leveraged to anticipate nanomaterial behavior and toxicity before clinical translation (Halappanavar *et al.,* 2019). Moreover, biocompatible and biodegradable nanomaterials—such as polymeric nanoparticles, lipid-based carriers, and naturally derived materials—are being prioritized to reduce systemic toxicity and environmental impact (Hou *et al.,* 2021; Poon *et al.,* 2020). On the regulatory front, there is a growing consensus on the need for harmonized guidelines, including nanoparticle-specific characterization, risk assessment protocols, and post-market surveillance mechanisms. As the field matures, collaboration among material scientists, toxicologists, bioinformaticians, and regulatory agencies will be crucial for translating nanotechnology into safe and effective applications in human health.

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