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Organic Nanoparticle Genotoxicity: Current Understanding and Future Testing Needs

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ABSTRACT

***Introduction**: Organic nanoparticles derived from biocompatible materials like chitosan, alginate, and lipids have garnered immense interest for drug delivery, bioimaging, and other biomedical applications. However, as their use rapidly expands, a comprehensive evaluation of their potential genotoxicity is crucial to ensure safe implementation. **Aims**: This review provides an in-depth analysis of the genotoxic risks associated with these organic nanoparticles. **Method**: The review elucidates how the unique physicochemical properties of organic nanoparticles can induce genetic damage through mechanisms such as direct DNA binding, oxidative stress, inflammation, and impairment of DNA repair pathways. Importantly, this genotoxicity can occur even in the absence of overt cytotoxicity, leading to heritable mutations and long-term adverse effects like cancer and reproductive abnormalities. A critical assessment of established and emerging genotoxicity testing methods, including their strengths, limitations, and opportunities for standardization, is presented. **Result**: The review synthesizes findings from existing in vitro and in vivo studies, revealing the contrasting genotoxic profiles of different organic nanoparticle formulations and exposure scenarios. Furthermore, the review provides insights into the multifaceted factors influencing nanoparticle genotoxicity, guiding the strategic engineering of safer designs. This comprehensive analysis underscores the pivotal importance of rigorous genotoxicity screening in the responsible development of organic nanomaterials. **Conclusion**: By harmonizing their innovative capabilities with a commitment to genetic integrity, this review paves the way for realizing the vast potential of organic nanoparticles while safeguarding human and environmental health.*

***KEYWORDS**: Chitosan, DNA damage, genotoxicity, liposomes, organic nanoparticles.*

INTRODUCTION

The past decade has witnessed rapidly growing interest in developing and applying organic nanoparticles fabricated from bio-based materials including polysaccharides, proteins, biodegradable polymers, and lipids

(Rao & Geckeler, 2011). Compared to traditional inorganic nanoparticles, organic nanoparticles offer unique advantages such as biocompatibility, biodegradability, and typically lower toxicity (Din et al., 2017). Their nano-scale size coupled with tailored surface properties gives organic nanoparticles great promise for wide-ranging applications from drug delivery to bioimaging to cosmetics (Gupta et al., 2016; Honary & Zahir, 2013; Paolicelli et al., 2009).

Among organic nanoparticles, chitosan, alginate, and liposomal systems have been particularly widely explored and applied given their assets. Chitosan, a linear polysaccharide derived from crustacean shells, can be assembled into nanoparticles through methods including ionic gelation, covalent crosslinking, emulsion droplet coalescence, and polyelectrolyte complexation (Divya et al., 2014). Cationic chitosan nanoparticles exhibit mucoadhesion, permeation enhancement, efflux pump inhibition, and tight junction opening effects ideal for mucosal drug delivery (Amin & Boateng, 2022; Ways et al., 2018). Alginate, a naturally occurring anionic polymer usually obtained from brown algae, can also be ionically crosslinked into hydrogel nanoparticles using calcium cations or cationic polymers (Tønnesen & Karlsen, 2002). Alginate nanoparticles leverage biocompatibility, relatively low toxicity, structural stability, and controlled drug release capabilities for pharmaceutical applications (Pawar & Edgar, 2012). Liposomes are vesicular structures comprised of lipid bilayers surrounding an aqueous core (Akbarzadeh et al., 2013; Bozzuto & Molinari, 2015; P. R. Kumar & Vijaya, 2020). Typical constituents of liposomes include phospholipids like phosphatidylcholines, cholesterol, and polyethylene glycol (PEG). Phospholipids found in eggs and soybeans, such as sphingomyelins, constitute the primary components of liposomes. The lipid bilayer consists of phospholipids and cholesterol, while PEG can decorate the outer surface. Hydrophilic drugs can be captured in the core whereas hydrophobic agents can be loaded into the lipid layers (Inglut et al., 2020; Sercombe et al., 2015). Liposomes have been extensively utilized in fields such as cancer treatment, delivering genes, vaccines, combating microbial infections, and other applications (Bozzuto & Molinari, 2015). While chitosan, alginate, and liposomal nanoparticles are increasingly used due to their biocompatibility, low toxicity, and functionality, their potential health and environmental impacts require further investigation given their expanding applications (H. Lu et al., 2021; Patra et al., 2018). Nanoparticles can elicit distinct effects from bulk materials given their greater surface area per mass and unique nanoscale interactions. Although the organic components are considered safe in bulk quantities, the nanoform toxicity warrants dedicated examination since nanoscale properties may differ from bulk. Conventional toxicity assays

based on bulk safety profiles could overlook key nanoparticle-specific hazards. Even for generally benign organic substances, thorough profiling of their nanoforms is essential to ensure safe implementation as use expands and to prevent unintended consequences. A comprehensive evaluation of the nanotoxicology of these organic nanoparticles is therefore critical moving forward (Thai et al., 2020). Numerous research studies have extensively examined the toxic effects of nanomaterials, including organic nanoparticles. Experts in the field have provide recommendations, tests, and techniques to develop methods for evaluating genotoxicity in nanomaterials. These recommendations aim to tackle the challenges nanomaterials pose and offer guidance for meaningful genotoxicity assessment. Assessing genotoxicity constitutes a pivotal endpoint in the evaluation of nanoparticle toxicity. It is of utmost significance in ascertaining the safety of compounds and materials, particularly organic nanoparticles. The potential of genotoxic agents to inflict harm on DNA material, inducing mutations and chromosomal alterations, is a significant concern. Such changes can disrupt cell function with far-reaching consequences for human health and reproductive abilities (Elespuru et al., 2018; Shukla et al., 2021). However, only some studies have comprehensively evaluated the genotoxic potential of organic nanoparticles.

In this review, we have thoroughly analyzed the existing body of research regarding the genotoxicity of promising organic nanoparticles: chitosan, alginate, and liposomal. We aim to summarize the significant in vitro and in vivo genotoxicity studies conducted while highlighting areas where further research is needed to fill existing knowledge gaps between the magnificent features of using nanotechnology in drug delivery and nanotoxicology. A thorough understanding of the genotoxic effects of these widely used nanomaterials will enable safer design and more responsible application. Comprehensive genotoxicity assessment of organic nanoparticles is essential to realize their promise while safeguarding human and environmental health. We performed a systematic search of several databases, including PubMed, ScienceDirect, and Google Scholar, using keywords such as "organic nanoparticles," "chitosan," "alginate," "liposomes," "genotoxicity," "DNA damage," and "mutagenesis." Additional relevant studies were identified through citation searching. We reviewed original research articles published in Scopus within the last ten years.

UNRAVELING THE ANIGMATIC PHYSICOCHEMICAL INTERPLAY OF ORGANIC NANOPARTICLES

The intricate physicochemical properties of organic nanoparticles orchestrate their multifaceted interactions within the complex biological milieu (Figure 1). Parameters such

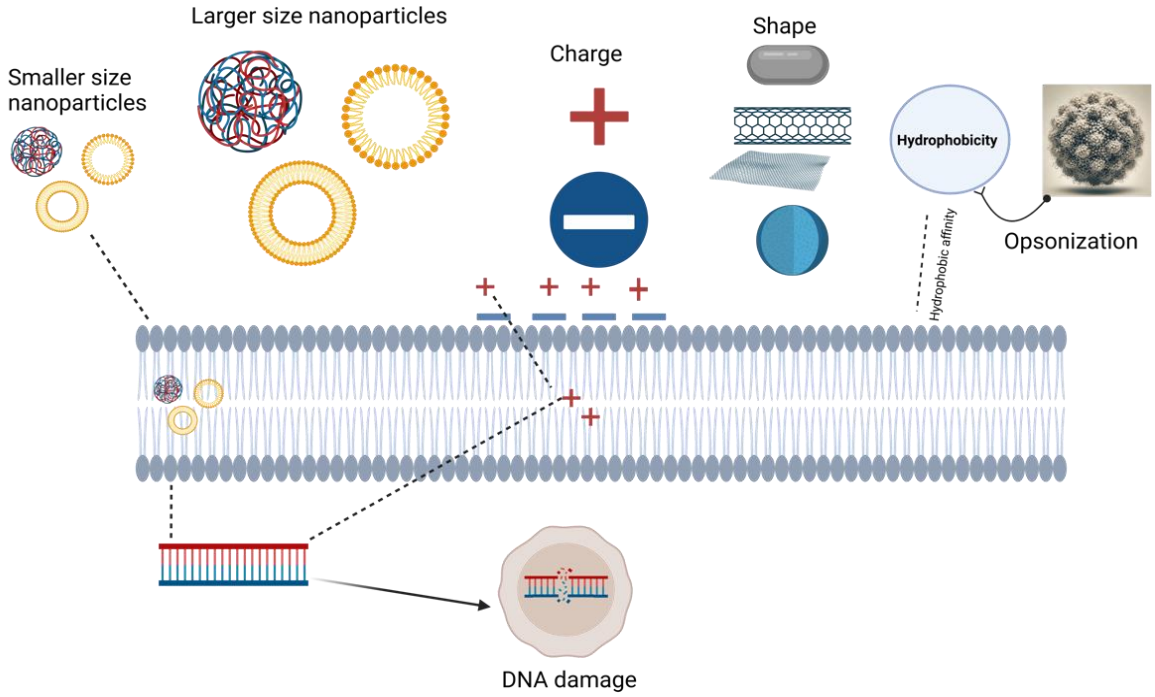

Figure 1. Influence of physicochemical properties on nanoparticle interactions with biological membranes.

as size, surface charge, hydrophobicity, molecular composition, and shape profoundly influence critical behaviors, including cellular uptake, biodistribution, immune interactions, toxicity, and functionality. These characteristics dictate how nanoparticles navigate biological systems, offering insights into their therapeutic potential and safety profiles (Abbasi et al., 2023; Blanco et al., 2015).

SIZE AS A DETERMINAT OF FUNCTIONALITY

Nanoparticle size plays a pivotal role in determining their functionality and interactions within biological systems, directly influencing therapeutic efficacy and toxicity profiles. Smaller nanoparticles, owing to their larger surface area-to-volume ratio, exhibit enhanced reactivity. This increased surface reactivity often results in elevated production of reactive oxygen species (ROS), which can induce oxidative stress and cellular damage (Abbasi et al., 2023). The ability of nanoparticles to penetrate cellular membranes is also highly size-dependent. Nanoparticles under 100 nm are efficiently internalized via endocytosis, enabling them to interact closely with intracellular components. Furthermore, exceptionally small nanoparticles, typically below 10 nm, can cross the nuclear membrane, raising concerns about potential interference with DNA and other vital biomolecules. For instance, studies on gold nanoparticles (AuNPs) have demonstrated size-dependent intracellular localization, with AuNPs measuring 10–16 nm predominantly localizing in the cytoplasm, while those smaller than 6 nm are capable of penetrating the cell nucleus.

This nuclear entry of smaller nanoparticles may contribute to increased toxicity due to potential interference with DNA and other critical nuclear biomolecules (Huo et al., 2014). The biodistribution of nanoparticles is similarly dictated by their size, with smaller nanoparticles demonstrating greater systemic mobility. Their ability to navigate through the body and cross biological barriers, such as the blood-brain barrier, enhances their potential for therapeutic applications but also increases the risk of systemic toxicity due to widespread tissue interaction (Prabha et al., 2016). Conversely, larger nanoparticles, typically exceeding 200 nm, may evade renal clearance due to their size but face heightened recognition and clearance by macrophages. This immunological response not only reduces their bioavailability but also raises the likelihood of inflammatory reactions, which may limit their clinical utility (Parmar et al., 2022). In biomedical applications, nanoparticles in the size range of 100–200 nm have been shown to effectively leverage the enhanced permeability and retention (EPR) effect. This phenomenon, prominent in tumor vasculature with leaky endothelial structures, allows for preferential accumulation of nanoparticles at tumor sites, thereby enhancing therapeutic delivery (J. Kim et al., 2023). However, while this size range balances systemic circulation and tumor targeting, it remains crucial to consider the trade-offs associated with size-dependent reactivity and biodistribution. The intricate relationship between nanoparticle size, reactivity, biodistribution, and cellular interactions underscores the importance of precise size optimization. Achieving this requires a holistic approach that accounts for the interplay between therapeutic efficacy and safety, ensuring nanoparticles are tailored to their intended biomedical application while minimizing adverse effects.

SURFACE CHARGE DYNAMIC

Surface charge significantly impacts cellular uptake and interactions. Cationic organic nanoparticles, such as those derived from chitosan or cationic lipids, exhibit a strong affinity for negatively charged cell membranes, promoting cellular internalization (Lohani et al., 2014). However, this electrostatic attraction may destabilize cellular membranes, enabling undesirable interactions with genetic material and potentially inducing genotoxic effects. Tailored surface modifications are thus essential to enhance nanoparticle functionality while minimizing adverse effects.

HYDROPHOBICITY AND ITS BIOLOGICAL IMPLICATION

Hydrophobicity exerts a multifaceted influence on nanoparticle interactions. Increased hydrophobicity can enhance plasma protein adsorption, promoting opsonization and clearance by the mononuclear phagocyte system (Owens & Peppas, 2006). Conversely, hydrophobicity stabilizes lipid-based nanoparticles, such as liposomes, by reinfor-

cing the integrity of their bilayer structures. Striking an optimal balance between hydrophobicity and structural stability is crucial for improving therapeutic efficacy while minimizing clearance and toxicity.

MOLECULAR COMPOSITION AND BIOCOMPATIBILITY

The molecular composition of organic nanoparticles governs their biocompatibility, degradability, and functional properties within biological environments (Elsabahy & Wooley, 2012). These characteristics are influenced by the specific organic materials used, such as polymers, lipids, proteins, or carbohydrates. For instance, alginate-based nanoparticles, derived from natural polysaccharides, have been extensively studied for their biocompatibility and biodegradability, showcasing potential suitability for drug delivery applications (Thomas & Latha, 2023). Surface functionalization enhances the biocompatibility of organic nanoparticles by improving stability, reducing immunogenicity, and enabling targeted delivery, as seen with polyethylene glycol (PEG) coatings that prolong circulation and minimize immune recognition (Sanità et al., 2020). Additionally, biodegradable polymers like polylactic acid (PLA) and polylactic-co-glycolic acid (PLGA) ensure safe degradation into non-toxic byproducts, facilitating their elimination from the body.

THE IMPORTANCE OF ASSESSING NANOPARTICLE GENOTOXICITY BEYOND CYTOTOXICITY

Cytotoxicity and genotoxicity represent two distinct forms of nanoparticle-induced toxicity, though both can impair cellular health. Cytotoxicity refers to direct nanoparticle damage to the cell that causes death through necrosis or apoptosis (Bhattacharya et al., 2011). Genotoxicity, on the other hand, refers specifically to nanoparticle-induced damage to DNA, including strand breaks, oxidation, and adduct formation (Kohl et al., 2020). Critically, genotoxic effects can be induced at non-cytotoxic doses and without significant cell death (Lewinski et al., 2008; Shukla et al., 2021). Unlike overt cytotoxicity, which leads to cell death, sub-lethal DNA damage induced by genotoxic exposures can become heritable mutations if the cells do not adequately repair the effects. The mutations may eventually manifest as cancer if oncogenes are activated, or tumor suppressor genes are inactivated (Evans et al., 2004). Genetic damage can also result in reproductive effects if germ cells are impacted (Eastmond et al., 2009).

Therefore, even without immediate cell death, the DNA damage caused by genotoxic compounds can produce adverse effects by altering the genetic code. This underscores the importance of evaluating genotoxicity early in the safety screening process for new compounds like engineered nanoparticles, separate from traditional cytotoxicity tests

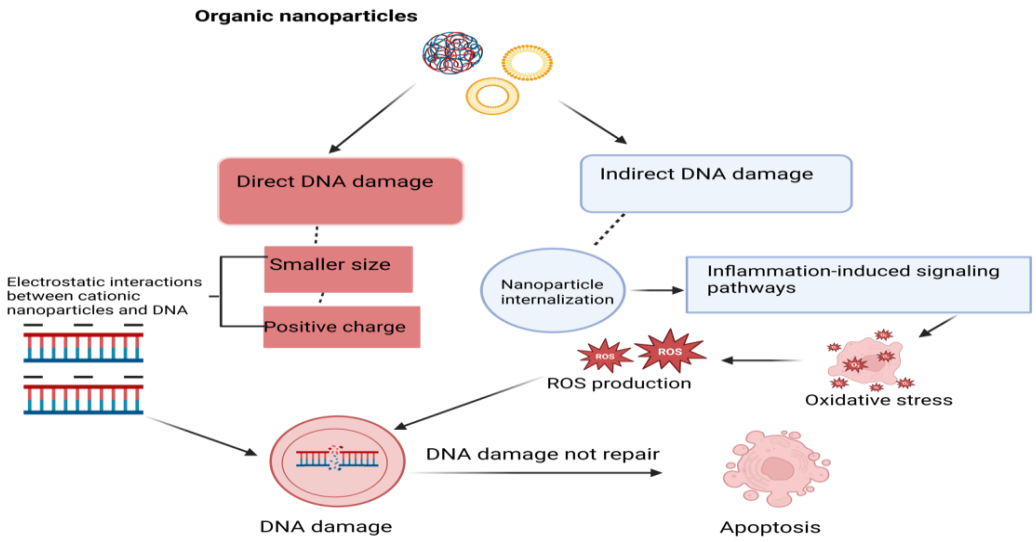

Figure 2. Mechanisms of organic nanoparticle-induced genotoxicity

(Shah et al., 2013).Some nanoparticles have demonstrated genotoxic effects through oxidative DNA damage and chromosome abnormalities without necessarily impacting short-term cell viability (Gonzalez et al., 2011; Kermanizadeh et al., 2013). Elucidating the properties that drive genotoxic vs. cytotoxic responses will assist in designing safer nanoparticles. Thorough genotoxicity assessments are vital for identifying potential long-term genetic hazards associated with nanoparticle exposures.

MECHANISM UNDERLYING GENOTOXICITY OF ORGANIC NANOPARTICLES

While many organic nanoparticles demonstrate low genotoxic potential, some nano formulations can elicit genetic toxicity through mechanisms involving direct DNA damage, indirect DNA damage, oxidative stress, apoptosis, and inhibition of DNA repair as shown in Figure 2.

Direct DNA damage can occur when nanoparticles physically interact with and disrupt the structure of DNA. Cationic nanoparticles exhibit affinity for binding to the phosphate backbone of DNA, causing deformations, strand breaks, and denaturation (V. Kumar et al., 2017; Liang et al., 2018; M. Singh, 2021). For instance, chitosan nanoparticles were found to directly bind and condense DNA in vitro (Rivera Gil et al., 2010). Nanoparticles may also directly interfere with replication and transcription of DNA. Indirect DNA damage stems from nanoparticle interactions with other cell components that secondarily cause genetic damage. Nanoparticles can prompt inflammatory responses and cell signalling disruptions leading to DNA breaks (Cinat et al., 2021; J. Liu et al., 2022; Z. Ye et al., 2021). For example, fullerenol nanoparticles were shown to induce TNF-α and IL-1β expression, correlating with DNA damage in vitro (L. Ye et al., 2021).

Nanoparticle-induced mitochondrial injury can initiate apoptotic signaling cascades that ultimately damage nuclear DNA. This secondary genotoxicity stems from mitochondrial oxidative stress and loss of structural integrity. Specifically, nanoparticles that permeate mitochondria can directly elicit lipid peroxidation and impair membrane potential. This mitochondrial disruption leads to excessive reactive oxygen species generation. Additionally, cytochrome c leakage from damaged mitochondria can activate cytosolic apoptosis pathways (Buoso et al., 2022; Tirichen et al., 2021; Yu et al., 2020). A relevant example is seen with zinc oxide nanoparticles in human HepG2 liver cells. After 12 hours of exposure to 14-20 μg/mL zinc oxide nanoparticles, HepG2 cells displayed mitochondrial dysfunction and oxidative stress. This was evidenced by decreased membrane potential, increased reactive oxygen species, and lipid peroxidation in the mitochondria. The zinc oxide nanoparticle-mediated mitochondrial damage also reduced the ratio of anti-apoptotic Bcl-2 protein compared to pro-apoptotic Bax protein in the HepG2 cells. This imbalance shifted the HepG2 cells towards apoptotic cell death signaling. Ultimately, the zinc oxide nanoparticle-induced mitochondrial oxidative injury and apoptosis activation promoted downstream nuclear DNA fragmentation (Sharma et al., 2012). This study highlights how nanoparticle interactions with mitochondria can indirectly elicit genotoxic effects through oxidative stress and apoptotic signaling cascades.

Oxidative stress is a key mechanism where excess intracellular reactive oxygen species and resulting inflammation degrades DNA bases, sugars, and histones (N. Singh et al., 2009). This leads to mutations and strand breaks. Nanoparticles may induce programmed cell death or apoptosis, preceding secondary necrosis and DNA damage through downstream apoptosis signalling rather than direct DNA interactions (Nazarparvar-Noshadi et al., 2020; Sharma et al., 2012). Finally, some types of nanoparticles can inhibit DNA repair pathways like base excision repair, nucleotide excision repair, homologous recombination, and non-homologous end joining that resolve DNA damage (Carriere et al., 2017). This allows mutations to accumulate. Elucidating the predominant genotoxicity mechanisms for different organic nanoparticle formulations will enable engineering approaches to mitigate their potential mutagenicity and improve safe design.

ASSESSMENT OF METHODS FOR GENOTOXICITY

Nanoparticles have unique properties that make them useful in various fields, including medicine, biotechnology, and material sciences. However, their potential toxicity and genotoxicity must be evaluated to ensure their safe use. Several in vitro and in vivo methods are used to assess the genotoxic potential of

Table 1. Advantages and limitations of genotoxicity assays for nanoparticle safety screening

Assay	Principle	In vitro/In vivo	Advantages	Limitations	References
Comet assay	Measures DNA strand breaks in individual cells based on increased migration of broken DNA in an electrophoretic field, visualized by fluorescence microscopy	Both	Rapid, simple, versatile, inexpensive, sensitive for detecting DNA damage and repair	Prone to inter-laboratory variability in results due to differences in experimental conditions. Standardization of protocols and evaluation criteria is crucial.	(Collins, 2004; Cordelli et al., 2021; Y. Lu et al., 2017)
In vitro comet assay	Uses cultured mammalian cells	In vitro	Provides mechanistic insights, amenable to high throughput screening	Lacks in vivo metabolic activation, less physiologically relevant	(Collins, 2004; Cordelli et al., 2021; Y. Lu et al., 2017)
In vivo comet assay	Uses cells isolated from animal models	In vivo	Accounts for ADME processes, more physiologically relevant	More time consuming, technical challenges in cell isolation	(Vidya et al., 2015)
Micronucleus assay	Detects chromosomal damage leading to formation of micronuclei containing chromosome fragments or whole chromosomes	Both	Well validated, widely accepted, amenable to automation	Non-specific, cannot detect structural or numerical chromosomal aberrations	(Kirsch-Volders et al., 2003; Sommer et al., 2020)
In vitro micronucleus assay	Uses cultured mammalian cells	In vitro	More rapid, higher throughput screening capabilities	Lacks in vivo metabolic activation	(Kirsch-Volders et al., 2003; Sommer et al., 2020)
In vivo micronucleus assay	Analyzes micronuclei in erythrocytes isolated from animal models	In vivo	Analyzes micronuclei in erythrocytes isolated from animal models	More time consuming, requires animal models	(Kirsch-Volders et al., 2003; Sommer et al., 2020)
Fast halo assay	Measures DNA unwinding from single strand breaks based on halo of unwound DNA from immobilized cells	In vitro	Rapid, simple, high throughput screening capabilities	Newer assay, validation still underway. Detects only strand breaks. Not fully automatic interpretation results	(Sestili, 2009; Sestili et al., 2006, 2017)

nanoparticles, such as single-cell gel electrophoresis (comet assay), micronucleus assay, and fast halo assay, as summarized in Table 1.

The comet assay, also termed single-cell gel electrophoresis, is a simple yet sensitive technique for evaluating DNA damage including strand breaks and crosslinks at the

individual cell level. It operates by analyzing the electrophoretic migration of negatively charged DNA fragments through an agarose gel matrix. Briefly, cells are suspended in low melting point agarose and pipetted onto microscopic slides pre-coated with normal melting agarose. After lysis and DNA unwinding under alkaline conditions, electrophoresis is performed, allowing broken DNA strands to migrate outwards from the cell nucleus toward the anode. The slides are then stained with fluorescent DNA intercalating dyes. Damaged cells exhibit a characteristic comet shape under fluorescence microscopy, with a distinct head containing intact DNA and a tail of migrated fragmented DNA. The proportion of DNA in the tail region correlates with the extent of damage. The comet assay can be coupled with specific enzymes to reveal particular lesions like oxidative adducts, thereby elucidating genotoxic mechanisms. Thus, it enables robust quantification of DNA damage at the single-cell level using minimal sample inputs (Collins, 2004; Cordelli et al., 2021; Y. Lu et al., 2017). Originally developed to detect double-strand breaks under neutral conditions, subsequent optimizations established alkaline conditions better suited for assessing low levels of single and double strand breaks relevant to genotoxicity (Shukla et al., 2021). The in vivo version accounts for absorption, distribution, metabolism, and excretion processes, contributing more physiologically relevant assessments (Vidya et al., 2015). For instance, an in vitro comet assay exposing human blood cells to cationic lipopeptide nanoparticles showed significant DNA damage after 1 hour of exposure, but no effects after 3 hours. Complementarily, an in vivo version found DNA damage in the liver, lung, and kidney of mice 24 hours to 14 days after injecting cationic lipopeptide nanoparticles. The kidney exhibited particular susceptibility to persistent genotoxicity. Such studies highlight the value of the sensitive comet assay for accurately determining nanoparticle genotoxic potential (Zhanataev et al., 2020).

The micronucleus assay is a commonly utilized technique for evaluating genotoxicity, including that caused by nanoparticles. It works by detecting the formation of micronuclei in cells. Micronuclei are small DNA fragments encapsulated in nuclear membrane that can arise from chromosome fragments or whole chromosomes that fail to properly migrate during cell division (Kirsch-Volders et al., 2003; Sommer et al., 2020). The assay can be performed in vitro using cell lines or primary human lymphocyte cultures. It can also be conducted in vivo using bone marrow or peripheral blood samples from animal models. Micronuclei are then visualized and scored under the microscope or via flow cytometry. This assay is sensitive and can identify both clastogenic effects involving structural chromosomal damage as well as aneugenic effects that alter chromosome number (Kohl et al., 2020; Kuo et al., 2022). Previous studies applied the in vitro micronuc-

leus assay in TK6 lymphoblastoid cells, lymphocytes from human volunteers, and bone marrow erythrocytes from rats exposed in vivo. The micronucleus assay in TK6 lymphoblastoid cells showed that, on average, titanium dioxide nanoparticles (NPs) did not cause a rise in micronuclei. However, lymphocytes from 3 out of 13 human subjects exhibited significant increases in micronuclei, signifying genotoxic effects in these cells. The in vivo micronucleus assay was conducted on bone marrow erythrocytes from rats exposed to titanium dioxide NPs revealed no elevation in micronuclei in rat bone marrow erythrocytes (Kazimirova et al., 2019). By combining the strengths of both in vivo and in vitro approaches , researchers can obtain a more comprehensive reliable genotoxic result.

The fast-halo assay is a rapid, less expensive, and sensitive method for assessing genotoxicity, including that of nanoparticles. It can provide mechanistic information about the mode of action of genotoxicants. However, the comet assay is more sensitive than the fast-halo assay in detecting DNA damage. The assay operates at the single-cell level and relies on radial dispersion of the fragments of damaged DNA from intact nuclear DNA to form halo shapes. The fragmented DNA is separated by diffusion in an alkaline solvent and is stained, visualized using florescence microscope (Sestili, 2009; Sestili et al., 2006, 2017). The assay can be used as rapid genotoxicity screening and assessments.

These methods are well-established, relatively inexpensive, and technically straightforward, making them accessible for studying a wide range of nanomaterials. Importantly, they are recommended by organizations like the Organisation for Economic Co-operation and Development (OECD) for genotoxicity screening of chemicals and nanoparticles (Luan & Honma, 2022). However, these assays have limitations that must be addressed. Using multiple complementary methods on the same set of organic nanoparticles strengthens conclusions by overcoming the intrinsic weaknesses of individual assays (Araldi et al., 2015). Developing standardized protocols, automated analysis, and machine learning interpretation can improve throughput, reproducibility, and accuracy (Bryce et al., 2010; Møller et al., 2020). For instance, the Halo-J software enables semi-automatic quantification of fast halo , which can enhance reliability of results interpretation (Maurya, 2014). Combining these well-validated genotoxicity screening tools with emerging technologies will provide robust safety profiles to advance the sustainable development of organic nanoparticles.

POTENTIAL TOXICITY OF ORGANIC NANOPARTICLES

Numerous research studies have focused on assessing the effects of nanoparticles by utilizing cytotoxicity assays. However, we undertook an investigation to compile and

summarize the existing research that examines the damage to genetic material caused by organic nanoparticles. Table 2 presents an overview of studies that have explored the safety profile, overall toxicity, cytotoxicity, and genotoxicity of organic nanoparticles.

The toxicity profiles of organic nanoparticles showcase intriguing contrasts. Chitosan nanoparticles (CNPs) exhibit cytotoxic and genotoxic effects in specific models like zebrafish embryos, as evidenced by developmental abnormalities, DNA damage, and indirect oxidative stress (Hu et al., 2011). However, CNPs also appear safer in other models like Wistar rats (Elnaggar et al., 2015). The cationic nature of chitosan and its smaller nanoparticle size likely contribute to DNA binding and damage. Further genotoxicity testing is warranted to elucidate the structure-activity relationships governing chitosan nanoparticle-DNA interactions. Moreover, CNPs demonstrate perplexing toxicity profiles—while eliciting cytotoxicity in particular in vitro models, they induce organ-specific damage without cell death in organisms like zebrafish. Developmental neurobehavioral and hepatotoxic effects arise without overt embryo mortality (Abou-Saleh et al., 2019). Interestingly, at a concentration of 200 µg/mL, CNPs lack cytotoxic effects in TM4 cells as assessed by CCK8, but induce DNA damage at the same concentration (200 µg/mL) and cause disruption in blood-testis barrier (BTB) proteins (Sadaqa et al., 2024) . These findings highlight the limitations of traditional cytotoxic assessments, which could overlook chitosan's potential subclinical toxicity. Measuring cytotoxicity alone risks misjudging actual hazards if underlying genotoxic, epigenetic, or signaling disruptions persist. Elucidating chitosan's mechanism of organ damage despite non-cytotoxicity could inform improved screening approaches. Combining cytotoxic panels with exploratory genotoxic, transcriptomic, and high-content imaging assays may provide a more holistic perspective on chitosan nanoparticle safety. The absence of cytotoxicity does not necessarily denote nanomaterial safety; methodical toxicity testing should probe beyond cell viability to capture subtler indicators of tissue dysfunction. A comprehensive understanding of chitosan nanoparticle toxicity, encompassing both cytotoxic and non-cytotoxic effects, is crucial for ensuring their safe and responsible application. In contrast, alginate nanoparticles demonstrate a favorable safety profile with minimal toxicity. For example, zinc oxide-alginate nanocomposites (ZnO-Alg/NCMs) showed protective antigenotoxic effects against the mutagen mitomycin C in mice. Encapsulation of peptides in alginate also reduced cytotoxicity compared to the free drug. The biocompatibility of alginate likely contributes to the observed lack of genotoxicity. However, expanded genotoxicity testing with multiple assays could further confirm alginate nanoparticle safety.

Table 2. Evaluation of toxicity and genotoxicity of various organic nanoparticles.

Nanoparticles	Test Model	Methodology	Major Findings on Toxicity	Reference
Chitosan nanoparticles (CNPs)	Zebrafish embryos	Acridine orange staining, ROS detection, HSP levels, hatching rate	Dose-dependent toxicity in embryos including increased mortality. Smaller NPs more toxic. ROS production led to indirect DNA damage	(Hu et al., 2011)
CNPs	Zebrafish embryos	Morphological assessments	Dose-dependent developmental toxicity. NPs less toxic than normal chitosan particles.	(Wang et al., 2016)
CNPs	Mouse hematopoietic stem cells	MTT assay	Dose and size-dependent toxicity. Smaller NPs more toxic.	(Omar Zaki et al., 2015)
CNPs	Zebrafish embryos	Behavioral assessments, liver size	No cytotoxic effect observed, yet on the other hand neurobehavioral, and hepatotoxicity observed.	(Abou-Saleh et al., 2019)
CNPs	Zebrafish embryos	Swim behavior, muscle histology	Motor deficits and muscle damage with NPs. Tween-modified NPs also toxic.	(Yuan et al., 2016)
CNPs	The human hepatocellular carcinoma cell line (HepG2)	MTT, ALT levels	Time and dose-dependent cytotoxicity. Liver enzyme leakage indicates damage.	(Loh et al., 2010)
CNPs	Zebrafish liver cells	MTT, trypan blue	Membrane disruption and cytotoxic effect on liver cells.	(Chou et al., 2020)
CNPs	Wistar rats	Caspase-3, TNF-α	No brain toxicity with intranasal administration. NPs safe and reduced nasal irritation of drug.	(Elnaggar et al., 2015)
CNPs	Mouse Sertoli cell line (TM4)	CCK8 , Comet assay , FHA	No cytotoxic effect was observed from CNPs, even at high concentrations of 200 µg/mL. However, this high concentration (200 µg/mL) induced DNA damage and downregulation of blood-testis barrier (BTB) proteins.	(Sadaqa et al., 2024)
chitosan/alginate nanoparticles (Chi/Alg/S NPs)	Human embryonic kidney(HEK 293 cell line)	MTT assay	No cytotoxic effect observed from Chi/Alg/S NPs on HEK 293 cell line	(Zohri et al., 2021)
miltefosine-loaded alginate nanoparticles	Red blood cells (RBC).	cytotoxicity assay on red blood cells (RBC).	miltefosine-loaded alginate nanoparticles showed reduced toxicity compared to free miltefosine	(Spadari et al., 2019)
ZnO/alginate-nanocomposites (ZnO-Alg/NCMs)	Bone marrow cells and spermatocytes after in vivo exposure to mitomycin C with or without pretreatment	chromosomal aberrations	Pretreatment with ZnO-Alg/NCMs for 7 days prior to mitomycin C (MMC) caused a greater reduction in MMC-induced chromosomal aberrations in bone marrow cells and spermatocytes compared to alginate alone. ZnO-Alg/NCMs exhibited higher antigenotoxic activity than alginate, providing greater protection against MMC genotoxicity.	(Hamouda et al., 2021)
Aliginate nanoparticles loaded with ICD-85 peptide	Primary lamb kidney cells.	MTT assay , LDH assay	Encapsulation ICD-85 peptide to aliginate nanoparticles decreased cytotoxic effect of free ICD-85 peptide on primary lamb kidney cells	(Mirakabadi & Moradhaseli, 2013)

Table 2 continues**.** Evaluation of toxicity and genotoxicity of various organic nanoparticles.

Nanoparticles	Test Model	Methodology	Major Findings on Toxicity	Reference
Cationic liposomes (DOTAP: cholesterol)	Wistar rats	In-vivo comet assay in the lung and spleen	DNA strand breaks were increased in the lung and spleen, suggesting genotoxicity of cationic liposome	(Knudsen et al., 2015)
Cationic liposome (Phosphatidylcholine: Stearylamine: Cholesterol)	Mouse macrophage-like cell line RAW264.	DNA content analysis ,flow cytometry and western blotting	Cationic liposomes induced ROS-mediated p38 MAPK activation leading to caspase-8 cleavage/tBid formation and subsequent mitochondrial apoptotic signaling incuding cytochrome c release in the mouse macrophage-like cell line RAW264.7 which might contribute to potential indirect genotoxic effects in RAW264.7 cells	(Iwaoka et al., 2006)

Cationic lipids commonly used in gene delivery vectors exhibit concerning genotoxicity signals in some models. In the study by (Knudsen et al., 2015), the use of cationic liposomes composed of DOTAP (1,2-dioleoyl-3-trimethylammonium-propane) and cholesterol resulted in increased DNA strand breaks in the lung and spleen tissues of Wistar rats, as evidenced by the in vivo comet assay. This finding suggests that cationic lipids can induce direct genotoxic effects in vivo, potentially leading to genomic instability and carcinogenic risks. Furthermore, (Iwaoka et al., 2006) demonstrated that cationic liposomes composed of phosphatidylcholine, stearylamine, and cholesterol triggered a cascade of apoptotic signaling events in the mouse macrophage-like cell line RAW264.7. The cationic lipids induced reactive oxygen species (ROS) production, leading to the activation of the p38 mitogen-activated protein kinase (MAPK) pathway. This, in turn, triggered caspase-8 cleavage, Bid truncation (tBid formation), and subsequent mitochondrial apoptotic signaling, including cytochrome c release. While not directly assessing genotoxicity, this study suggests that cationic lipids can initiate oxidative stress and apoptosis, which may contribute to indirect genotoxic effects through the generation of reactive species and genomic instability. The genotoxic potential of cationic lipids is a significant concern, as it could compromise the safety and clinical translation of lipid-based gene delivery systems. Their cationic charge likely enables direct DNA interactions, similar to chitosan (Iwaoka et al., 2006; Knudsen et al., 2015). Further testing is required to elucidate their genotoxic mechanisms fully.

The previous table highlights the variability in the safety, cytotoxicity, and genotoxicity of organic nanoparticles like chitosan, alginate, and cationic lipids. Despite straightforward, inexpensive genotoxic assays, few studies have specifically quantified the genotoxic impacts of these organic nanoparticles, with most research emphasizing cytotoxicity evaluations. In contrast, inorganic nanoparticles like silica, silver, cerium dioxide, titanium dioxide, and iron oxide are

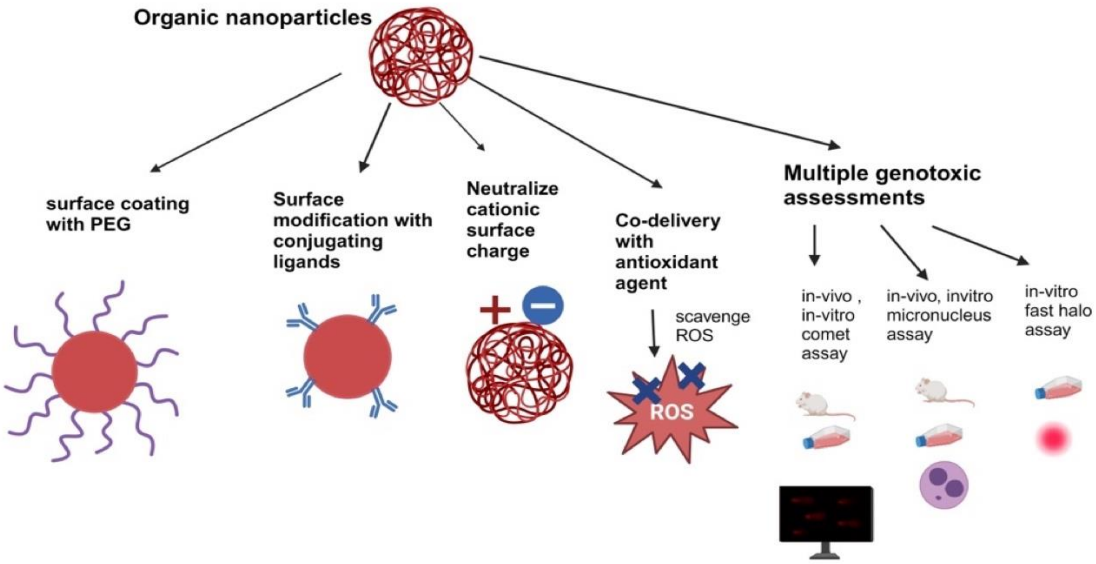

Figure 3. Schematic of strategies to reduce genotoxicity of organic nanoparticles

more comprehensively evaluated for genotoxicity (Magdolenova et al., 2012, 2015; Osman IF et al., 2011; Preaubert et al., 2016). This presents an apparent contradiction, as organic nanoparticles comprise biodegradable components expected to be safer than inorganic materials. Thus, traditional cytotoxic assays may be insufficient to determine organic nanomaterial toxicity, and thorough genotoxic profiling is recommended to confirm their safety.

GUIDING PRINCIPLES FOR MITIGATING GENOTOXICITY OF ENGINEERED ORGANIC NANOPARTICLES

The responsible development of organic nanoparticles for biomedical applications necessitates a proactive approach to mitigating their genotoxic potential. By strategically engineering their physicochemical properties and formulation parameters, the risk of genetic damage can be minimized while preserving their therapeutic efficacy. Several guiding principles can be implemented during the design and fabrication stages to enhance the safety profiles of these nanomaterials (Figure 3).

Surface modification and targeting strategies can significantly influence nanoparticle biodistribution, cellular interactions, and subsequent toxicity (Saravanan & Tippavajhala, 2022). Polyethylene glycol (PEG) coating, a widely employed strategy, can minimize nonspecific protein adsorption and cellular uptake, thereby reducing the likelihood of genetic insult (Jokerst et al., 2011; Van Haute et al., 2018). However, excessively high PEG densities can limit its protective effects by causing crowding and folding of the polymer chains. This reduces coating coverage and exposes more of the nanoparticle surface for binding. Therefore, the conformation and packing of the PEG coating strongly influence its ability

to shield nanoparticles and minimize cellular uptake effectively. A balanced density that allows sufficient surface coverage without folding or crowding is ideal to leverage the full benefits of PEGylation (Cahn & Duncan, 2022; X. Lu & Zhang, 2018; Ma et al., 2021; Shi et al., 2021; Suk et al., 2016). Alternatively, active targeting strategies, such as conjugating ligands (antibodies or peptides) that selectively bind to specific cells or tissues, can facilitate targeted delivery while limiting off-target accumulation in sensitive organs like bone marrow, where genotoxic effects may be more pronounced.

The surface charge of nanoparticles plays a pivotal role in mediating their interactions with biological components, including negatively charged DNA. Cationic nanoparticles exhibit a propensity to electrostatically bind and condense DNA, potentially leading to structural deformations and strand breaks (Zielińska et al., 2020). Conversely, neutral or slightly anionic surface charges can minimize such adverse interactions, thereby reducing the risk of direct genotoxicity. Intelligent selection of coating materials or incorporation of anionic polymers can effectively modulate the surface charge to mitigate genotoxic potential. Oxidative stress, a prominent mechanism underlying nanoparticle-induced genotoxicity, can be alleviated by incorporating antioxidant compounds into the nanoparticle formulation. Antioxidants like vitamin E, quercetin, and curcumin can scavenge reactive oxygen species (ROS) and suppress oxidative DNA damage (Habas et al., 2018; Q. Liu et al., 2023; Xie et al., 2022). Additionally, co-delivery of antioxidant enzymes, such as superoxide dismutase and catalase, can further bolster the nanoparticle's ability to mitigate oxidative stress and associated genetic insults (S. Kim et al., 2019; Singhal et al., 2013). Traditional cytotoxicity assessments alone may be insufficient to comprehensively evaluate the safety of organic nanoparticles. A multi-faceted approach, combining complementary in vitro and in vivo genotoxicity assays, is crucial for generating a comprehensive genotoxicity profile. Well-established techniques like the comet assay, micronucleus assay, and fast halo assay offer rapid, sensitive, and cost-effective means to probe DNA and chromosomal damage. Applying a battery of such assays during the early stages of nanoparticle development can identify potential genotoxic liabilities and guide the engineering of safer formulations.

Emerging computational approaches, including in silico modeling and predictive toxicology, hold promise for anticipating the genotoxic potential of organic nanoparticles prior to extensive experimental testing (Afantitis et al., 2020; Gajewicz et al., 2012; Halder et al., 2020). By leveraging advanced algorithms and machine learning techniques trained on existing genotoxicity data, these computational tools can rapidly screen and prioritize nanoparticle candidates based on their physicochemical properties and predicted biological interactions. While still an evolving

field, the integration of in silico approaches into the nanoparticle development pipeline can streamline the identification of safer designs and accelerate the responsible translation of these innovative nanomaterials.

By harmonizing these guiding principles, researchers and industries can strategically engineer organic nanoparticles that harness their therapeutic potential while safeguarding genetic integrity. This proactive approach, combining intelligent design strategies, rigorous genotoxicity screening, and emerging computational tools, paves the way for realizing the vast clinical potential of organic nanomaterials while prioritizing human and environmental health.

REGULATORY CONSIDERATION AND FUTURE DIRECTIONS

As innovative organic nanoparticles advance toward real-world applications, regulatory agencies are taking steps to evolve frameworks for evaluating their human and environmental impacts. Groups like the FDA have begun issuing guidance documents outlining considerations for the physicochemical characterization, toxicity testing, and risk assessment of nanomaterials used in regulated products (Doak & Dusinska, 2017; Dusinska et al., 2016). However, much work remains to develop standardized testing guidelines tailored to organic nanoparticles' unique properties and vast diversity. Critical priorities for regulatory bodies include expanding curated datasets, harmonizing test methods across regions, integrating emerging technologies like high-throughput assays, and in silico modeling collaborating with academia and industry. Adoption of formalized validation processes will be critical to qualify new methodologies for regulatory decision-making. Future directions should also promote the development of safe-by-design approaches incorporating hazard assessment and mitigation early in the nanomaterial lifecycle. Overall, regulators have an essential role in stimulating the generation of the evidence base required to translate organic nanoparticles from the benchtop to real-world implementation responsibly.

CONCLUSION

Organic nanoparticles demonstrate remarkable potential in biomedicine, particularly for drug delivery and bioimaging, due to their biocompatibility and functional versatility. However, this review underscores the need for a safe-by-design approach to address the nuanced risks posed by these materials at the nanoscale. While cytotoxicity assessments provide valuable insights into immediate toxic effects, they are insufficient to evaluate the long-term and subtle risks of genotoxicity, including DNA strand breaks and oxidative stress-induced damage, which may occur independently of cytotoxic effects. Advanced genotoxicity assays, such as comet, micronucleus, and fast halo techniques, combined with strategies like surface modifications and antioxidant incorporation,

are pivotal for mitigating these risks. Furthermore, integrating standardized methodologies with futural computational modeling strengthens the robustness of safety evaluations. By adopting a safe-by-design paradigm that comprehensively addresses cytotoxicity and genotoxicity, organic nanoparticles can be developed to maximize their therapeutic potential while prioritizing human health and safety.

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