

REVIEW

Perspectives in Nanotoxicity Studies for Carbon-Based Nanomaterials in 3D Platform

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ABSTRACT

The conventional two-dimensional (2D) in vitro cell culture system has been a staple in biological research for decades. However, due to numerous limitations, this strategy is being replaced by 3D cell culture, representing a significant advancement in cell culture techniques. The 3D culture has garnered substantial attention across various scientific fields due to its ability to replicate in vivo-like physiological conditions. In the same spirit, mimicking in vivo scenarios is crucial for assessing the adverse effects of materials, in particularly carbon-based nanomaterials. This review highlights recent advancements in cell culture techniques and various innovative 3D cell culturing strategies, and concurrently addresses the current status and limitations of cytotoxicological studies related to carbon-based nanomaterials. The application of 3D cell cultures in toxicological studies of carbon-based nanomaterials is discussed with an emphasis on the potential of these advanced techniques. Additionally, the review delves into the remaining challenges associated with strategies for assessing the toxicity of carbon nanomaterials in 3D cell culture platforms and explores future perspectives in this evolving field.

1 | Introduction

Carbon-based nanomaterials (CNMs) are referred to as novel nanoscopic-scale forms of carbon in nanomaterials that have been discovered over the last two decades. CNMs can be categorized into carbon nanotubes (CNTs), fullerenes, graphene and its derivatives, graphene oxide, nanodiamonds, carbon-based quantum dots, etc. From an evolutionary perspective, the development of carbon fibers in 1879 marked the first step in nanoscale carbon materials. Diamond fragmentation in 1888 and the isolation of detonation nanodiamonds in 1963 laid the foundational basis, followed by the discovery of fullerenes (1985) and carbon nanotubes (1991). These milestones opened

the door to the development of advanced carbon nanostructures, including carbon onions, carbon dots, and graphene, as illustrated in Figure 1.

Owing to distinct properties such as unique structural dimensions, and excellent mechanical, electrical, thermal, optical, and chemical properties, CNMs have been used in diverse areas of the scientific fields, including energy storage, catalysis, energy conversion, drug delivery, biosensors, DNA sequencing, artificial tissue engineering, electromagnetic shielding, and bioimaging, etc [1]. For instance, in the field of drug delivery, both CNTs and graphene have demonstrated indispensable potential applications for delivering drugs more efficiently and

Summary

- The 3D cell culture systems are demand of the hour, driven by the need for more physiologically relevant in vitro models for nanotoxicity assessment
- In this review, the priority of using 3D cell culture for in vitro toxicity studies of carbon-based nanomaterials has been systematically studied and discussed.

innovating therapeutic approaches, especially for cancer treatment, due to their properties of enhancement in surface area, high capacity of loading drugs, and surface with modifiable [2]. Also, recent literatures have revealed that CNMs such as CNTs, carbon nanofibers (CNF), and graphene have been used for cement composites, due to their enhanced surface area, mechanical strength, and electrical conductivity [3]. Even one recent study has advocated that the pivotal problems of heavy metals' toxicity and disposal can be replaced by mass production of CNMs [4].

However, these unique nanomaterials have faced difficulties in scalability, due to some unknown issues such as the toxicity of CNMs to human health when exposed to the environment, skepticism about the biocompatibility of medical applications, etc. Until now, a growing number of studies related to the nanotoxicology of CNMs on various cell types, tissues, and physiological systems have been intensively investigated, yet challenges persist due to controversial and inconsistent data reports. For instance, some earlier studies have reported that CNMs exhibit a lower toxicity profile in living systems compared to metal nanoparticles, including silica-based Cornell dots, as well as lipid- and polymer-based nanoparticles [5]. Also, some studies have demonstrated that CNMs are environmentally friendly and exhibit minimal toxicity in both in vivo and in vitro systems after functionalization [6, 7]. On the other hand, many studies have found that CNTs exhibit toxic effects on human MSTO-211H cells, A549 human pneumocytes, human lung epithelial H460 cells, and HeLa cell lines [8–11]. With the advent of three-dimensional (3D) cell culture techniques, cytotoxicological studies of CNMs also have taken new steps. Thus, reliable assessment and experimental data are important to monitoring the adverse environmental impacts of the wide use of carbon-based nanomaterials.

This review aims to elucidate in detail conventional studies involving in the adverse effects of CNMs in vitro, the advent of novel 3D cell culture techniques and their replacement of the 2D cell culture techniques, and their wide usage in ecotoxicological studies related to CNMs. Unlike the reviews in the literature on the cytotoxicity of CNMs platforms, which concentrate on only partial factors that trigger the toxicity of carbon-based nanomaterials in vitro, this study systematically elucidates, for the first time, previous studies related to the cytotoxicity of different types of CNMs in conventional 2D cell culture techniques, as well as recently updated toxicity studies related to novel 3D cell culture techniques. In addition to collecting information from a variety of sources, we present strategies and challenges for assessing the adverse effects of carbon-based nanomaterials in vitro using novel 3D cell culture

techniques. Also, we speculate on future perspectives of studies related to the adverse effects of CNMs in 3D cell culture, and aim to shed light on the next frontier in 3D cell culture for the toxicology of CNTs.

2 | Revolutionized in Vitro Culture Tools for Biomedical Research

The cell culture technique of maintaining live cells of interest separated from living tissue was first implemented by Wilhelm Roux in 1885. This technique is also known as conventional two-dimensional (2D) cell culture, which provides mechanical support for the cells during culturing through adherence to a flat surface such as a petri dish or polystyrene. There are a myriad of purpose of growing cells in culture such as (i) to investigate the normal physiology or biochemistry of cells, (ii) to screen the effects of various types of chemical drugs, (iii) to study artificial tissues through combination of various cell types for tissue engineering, (iv) to produce a broad range of cell products, specific proteins or viruses and essential biomolecules.

In the mid-20th century, cell culture techniques became most popular with the main advantages of consistency and reproducibility of results obtained from a batch of clonal cells [12, 13]. In general, tissue culture, organ culture, and viral culture are closely associated with the historical development and methods of cell culture. In the 1950s, scientists produced the polio vaccine, which was the first commercial product of animal cell culture. Moreover, another milestone of cell culture is the production of hybrid cells or hybridoma, which are capable of continuous production of a single type of antibody for diagnostic and therapeutic applications in 1975. The development of the vaccine and the culture of HeLa cells- the first human cells cloned in 1953- reformed cell culture research and paved the way for a broad range of applications.

The introduction of three-dimensional (3D) cell culture to Cell Biology has revolutionized the Biomedical sphere by adding a new dimension from the evolution line of “static to motional or dynamic” [14–16]. The motivation to work with cellular models that are physiologically relevant to living tissue has shifted scientists from cell monolayer to 3D cultures that is natural, in vivo-like system. The first landmark 3D cell culture model was developed by researchers in 1992 by using 3D organotypic structures to mimic breast structures [17]. As a foundation for biomedical and preclinical research, 3D culture mainstream approach is rapidly propagating to areas such as genomics, proteomics and molecular cell biology, and it is envisioned that it will have applications across a wider range of scientific fields.

3 | A Fabulous Innovation That Mimicking Microarchitecture and Functions of the Living Organ in Vitro

3D cell culture is defined as an artificial, highly dynamic microenvironment that allows cells to grow in vitro via interaction with their surroundings in all dimensions. 3D cell culture is a natural response to address the limitations of

conventional 2D monolayer cell culture. Several properties highlight the advantages of 3D cell culture, such as exhibiting in vivo-like surroundings and different gene expression compared to conventional cell cultures, behaving in a manner physiologically relevant to the whole living organs, serving as a more advanced model for deciphering cell behaviors, including migration, differentiation, survival, growth, and polarization [16, 18, 19]. Additionally, researchers have found that 3D cell cultures are more stable and have a longer lifespan compared to the classic methods [20]. To give an example, to better mimic a tumor microenvironment that consists of different processes of progression in cancer, such as growth, metastasis, migration, and chemotherapeutic resistance, more than 1000 research papers were published in a single year [21, 22] (Figure 2). In another example, scientists developed a 3D hydrogel hematological disease modeling system that mimics hematopoietic differentiation of iPSCs in Down syndrome-associated transient myeloproliferative disorder (TMD) and concluding consistency of TMD characteristics and hematological disease model systems for further study [31, 32].

As outlined in the literature, diverse methods have been employed to meet the growing demands of 3D cell culture. However, it is not rational to use a single 3D hydrogel-based system for hematopoietic differentiation, or modeling down syndrome-associated transient myeloproliferative disorder method as a panacea for all 3D cell culture, but it certainly depends largely on researcher's selection of an appropriate type of cell culture model according to their needs. In general, two different approaches can be used to generate 3D cell culture, including scaffold-based and scaffold-free techniques [33, 34]. In the scaffold-based approach, artificial or synthetic solid materials such as ceramics, metals, or polymers are being used, while polysaccharides, proteins, extracellular matrix-derived, acellular matrix, and hydrogel are being used in the natural scaffold approach [35]. For example, to facilitate efficient tissue vascularization and improve nutrient and metabolite exchange in engineered tissues, researchers have developed ceramic hollow fiber membranes integrated with 3D tissue models, which enable the separation of red blood cells and mononuclear cells for translational cell therapy studies [36]. Solid porous scaffolds fabricated from biodegradable polymers are widely used due to their porous structure and biocompatibility [37–39]. In addition, hydrogels are also popular scaffolding products, which can be natural, synthetic, or hybrid (natural and synthetic) forms [40–45]. Alternatively, spheroids, organoids, or self-assemblies are being adapted in scaffold-free technique. For instance, spheroids have been widely used in skin cancer research since 1944, when Holt Freter first investigated skin behavior using spheroid models [46]. In recent years, researchers have developed 3D melanoma spheroid cultures to further study

molecular mechanisms underlying disease pathology [47]. In addition, spheroids have been widely used to mimic characters of native tissue of different types of cancer in vitro [48–56]. Organoid technology holds great promise for 3D cell culture in many fields such as regenerative medicine, drug discovery, and precision medicine. Until now, organoids of intestine, stomach, liver, pancreas, lung, kidney, brain, and retina have been established using either adult stem cells or pluripotent stem cells [57–68].

4 | Status of in Vitro Cellular Models for Carbon-Based Nanomaterials Toxicity

In brief, biocompatibility refers to the capability of a material to be in contact with a living system without producing an adverse effect. Biocompatibility is related not only to the physical and chemical properties of a material, such as shape, size, and charge, but also to toxic substances arising from manufacturing processes and degradation products formed in the body. Biocompatibility can be examined in different effects through short-term contact and long-term contact between the material and the body. The biocompatibility of biomaterials is a vital indicator of evaluating the use of materials in the biomedical field that must be examined properly.

A diverse range of studies has been conducted to elucidate the mechanisms underlying the toxicity of carbon-based nanomaterials [69] (Figure 3). One proposed mechanism involves the permanent mechanical rupture of cellular or nuclear membranes due to the penetration of CNTs into the cellular environment. Evaluating this mechanism involves considering physical parameters such as the type of CNTs, length, diameter, synthesis method, catalysts, incubation time, dose of CNTs, and so on. Researchers have extensively investigated these factors over the years to understand their impact on cellular membranes. Another potential mechanism centers around the penetration of CNTs into cells, leading to an elevation in the levels of reactive oxygen species (ROS). ROS is implicated in a wide array of adverse effects in cells, including apoptosis, necrosis, cytochrome c release, oxidative DNA damage, reduced proliferation, inhibition of cell growth, and G2/M cycle arrest. To assess this mechanism, various biological analyses are employed, including MTT, WST-1, and MTS colorimetric assays for cell viability, lactate dehydrogenase (LDH) assays for evaluating cell membrane integrity [70], and flow cytometry and microscopy to study apoptosis or necrosis. Additionally, ATP production studies are conducted to examine mitochondrial dysfunction. The third mechanism involves genotoxic effects, defined as DNA damage induced by CNTs. Genotoxicity includes chromosome aberrations resulting from the interaction

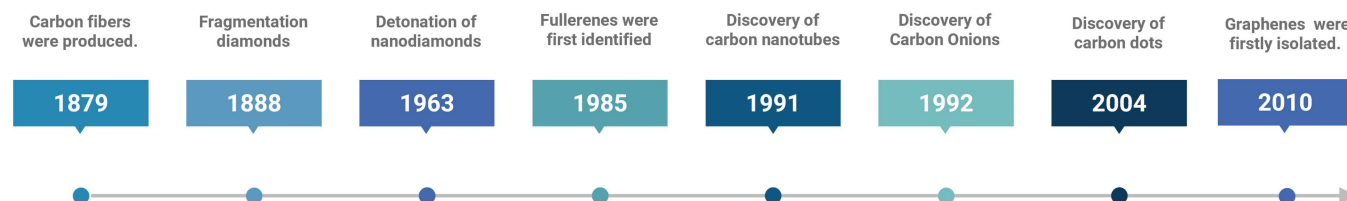


FIGURE 1 | The historical evolution of carbon-based nanomaterials (Created in BioRender).

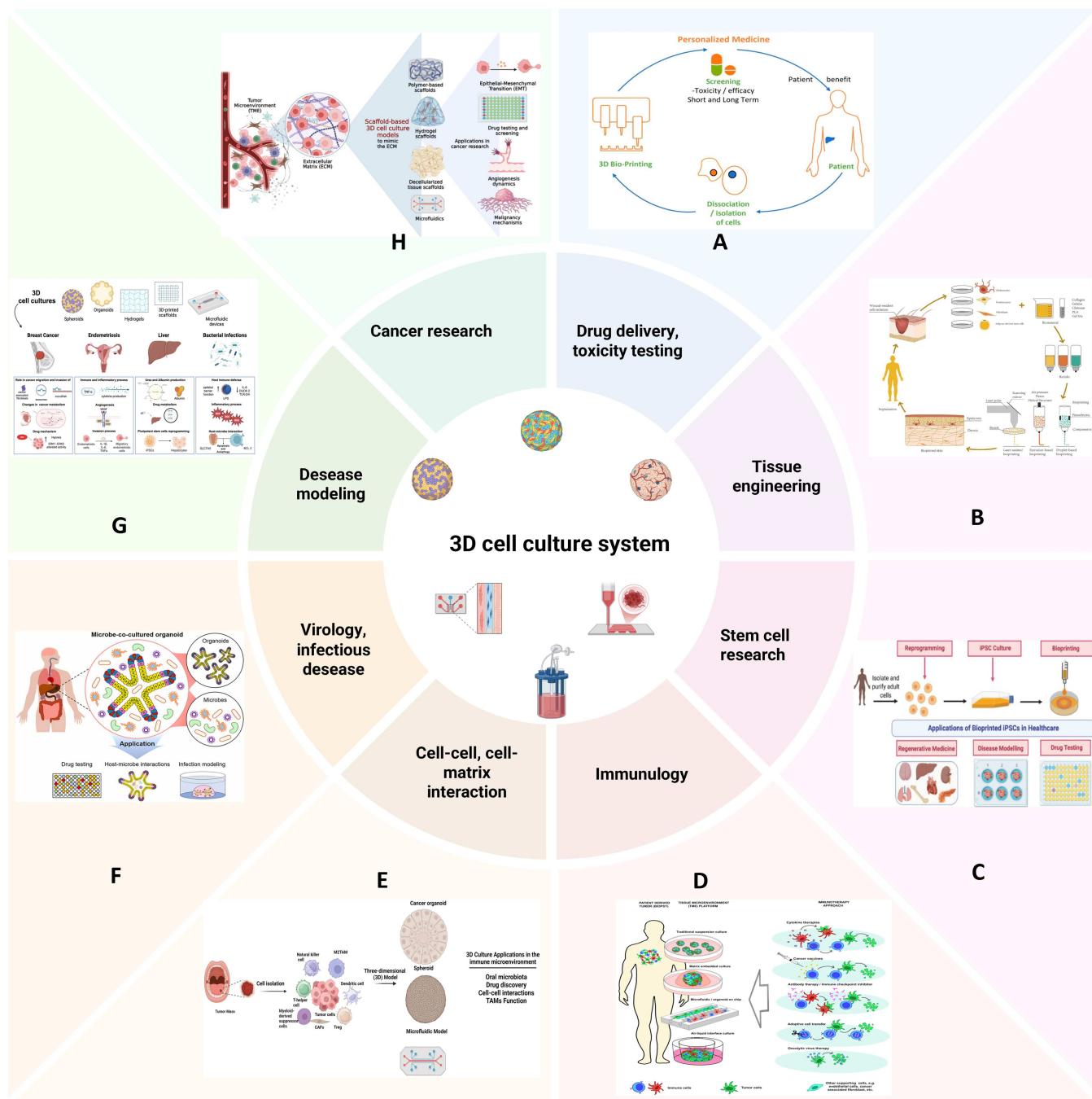


FIGURE 2 | Schematic illustration of 3D cell culture applications in drug delivery, toxicity testing, stem cell research, immunology, cell-cell and cell-matrix interactions, virology, infectious diseases, disease modeling, and cancer research (created with BioRender). Reproduced with permission: (A) from Ref. [23], CC BY 4.0; (B) from Ref. [24], CC BY 4.0; (C) from Ref. [25], CC BY-NC 4.0; (D) from Ref. [26], CC BY 4.0; (E) from Ref. [27] [E], CC BY 4.0; (F) from Ref. [28], CC BY 4.0; (G) from Ref. [29], CC BY 4.0; (H) from Ref. [30], CC BY 4.0.

of CNTs with cellular proteins, the impact of CNTs on the mitotic spindle, micronuclei formation, indirect DNA oxidation, and DNA breakage. Mitochondrial dysfunction can be assessed by studying ATP production for the respiratory control ratio. Inflammatory biomarkers such as IL-8(Interleukin-8), IL-6(Interleukin-6), and tumor necrosis factor can be evaluated using enzyme-linked immunosorbent assays (ELISA). Notably, researchers have tested approximately 80 different types of cell lines between 2000 and 2021 to investigate the cytotoxic effects of CNTs [71] (Figure 4).

The majority of toxicity assays for CNTs have been conducted using 2D in vitro models. Carbon-based nanomaterials exhibit various types based on their structures, encompassing carbon nanotubes, fullerene, graphene and its derivatives, graphene oxide, nanodiamonds, and carbon-based quantum dots [72] (Figure 5). The diversity in carbon-based nanomaterials allows researchers to explore a broad spectrum of structures and functionalities, each with unique properties and potential applications.

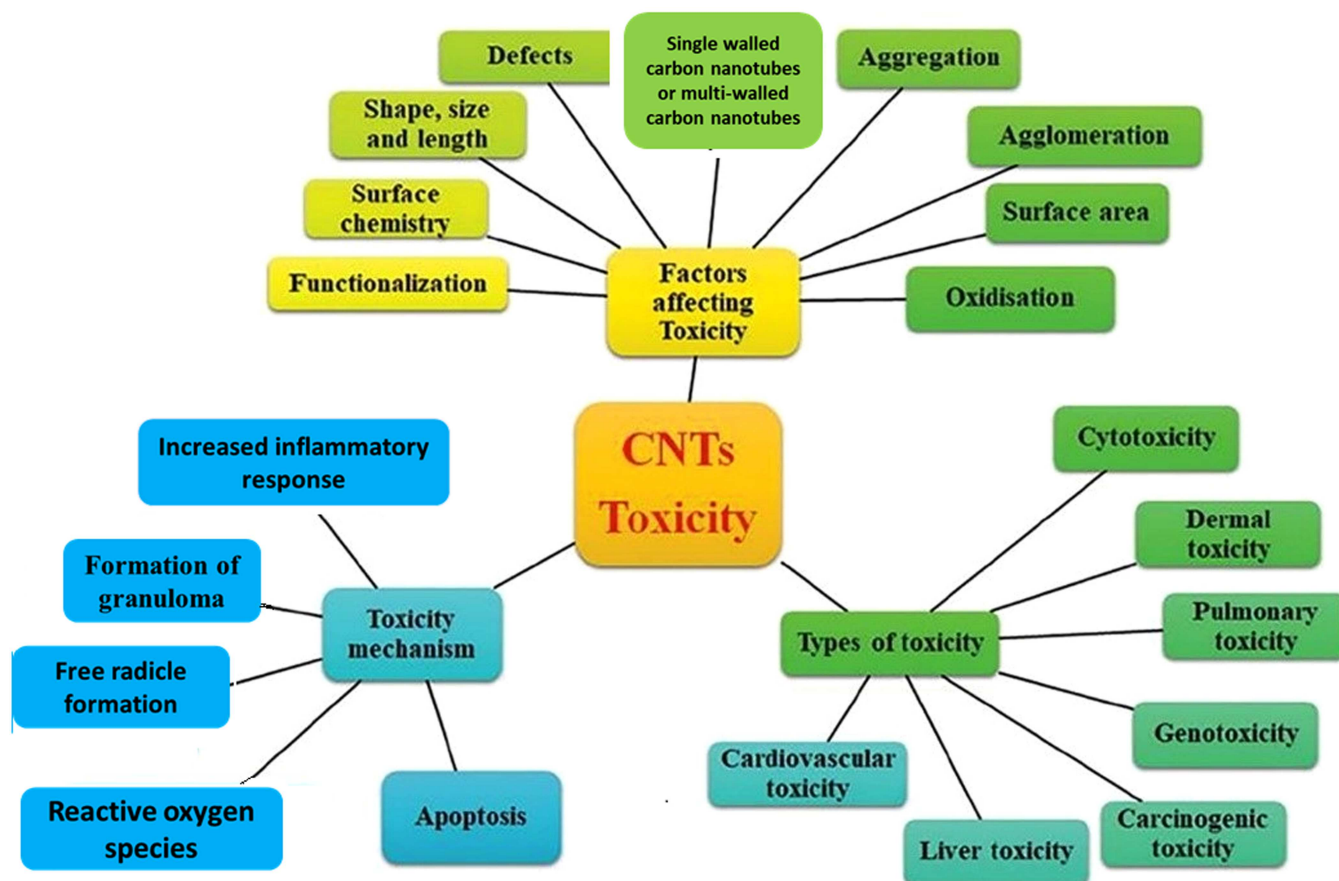
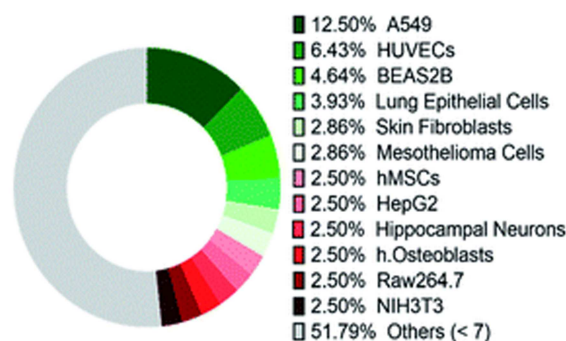


FIGURE 3 | CNTs toxicity mechanism, types and factors inducing the toxicity. (adapted from ref [69]).

A Type of cells



B Cytotoxicity assay

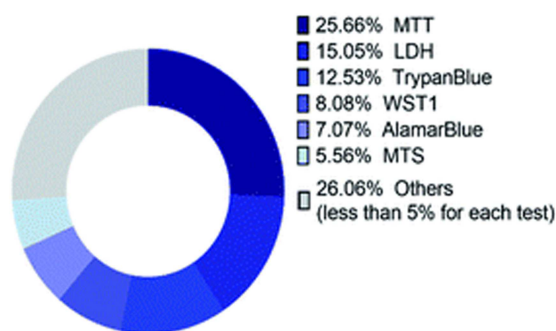


FIGURE 4 | Statistics for two crucial parameters vary greatly among in vitro examinations: cell types and applied cytotoxic test. (A) Cell types used in the studies included in the present review analysis; (B) tests used for evaluation of the CNTs toxicity in dispersion (adapted from ref. [71] with permission under CC BY-NC 3.0 license).

4.1 | Carbon Nanotubes

As a member of the fullerene family, carbon nanotubes (CNTs) are allotropes that have many superior features, such as high rigidity and extraordinary flexibility as well [73, 74]. CNTs can be divided into two major categories according to graphite cylinders' arrangements in their structure, such as single-walled carbon nanotube (SWNTs) and multi-walled carbon nanotubes (MWNTs) [75].

Within the tremendous potential application of carbon nanotubes (CNTs) in multiple industries [76] (Figure 6). They have not yet been fully applied to the biomedical sphere since the discrepancy of data [77, 78] related to the toxicologic studies of CNTs. For example, a bulk of data related to studies of cellular toxicity concerning MWCNTs exhibits significant inconsistency [79, 80]. One study reported that the toxicity of two types of MWCNTs did not contribute to the induction of apoptosis in

Various nanoforms of carbon allotropes

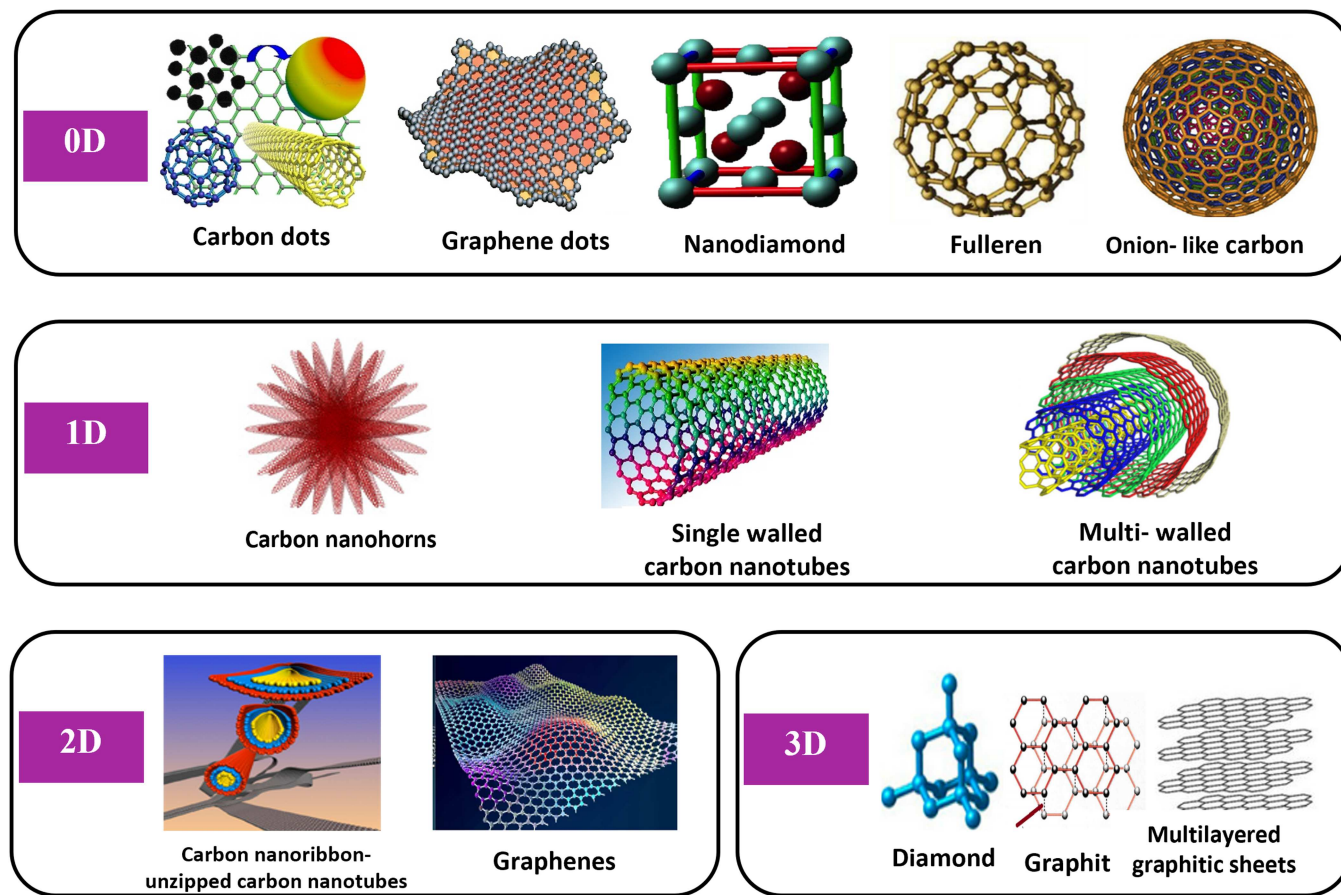


FIGURE 5 | Types Of Carbon Allotropes.

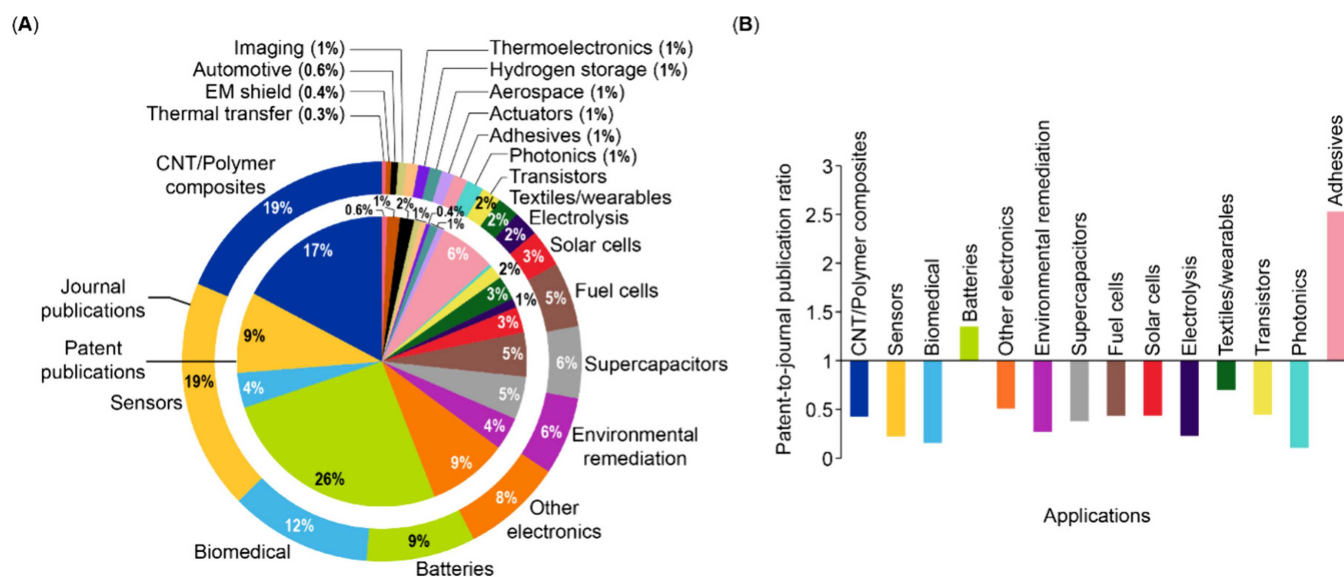


FIGURE 6 | (A) Pie chart showing the distribution of publications related to individual CNTs applications for journals (outer) and patents (inner). (B) Ratio of patents to journal publications for selected applications. Data includes journal and patent publications in the field of carbon nanotubes from the CAS Content Collection for the period 2003–2023 (adapted from ref. [76] with permission under CC BY 4.0 license).

RAW 264.7 cells [81]. In contrast, another study reached the opposite conclusion, demonstrating that acid-treated and taurine-functionalized MWCNTs induced cell death and impaired cellular phagocytosis [82]. In addition, different types of cells and macrophages respond to MWCNTs exposure in distinct ways. Concentration of 3–30 $\mu\text{g}/\text{ml}$ led to apoptosis of murine bone marrow-derived dendritic cells, while cytotoxic effect had not found in RAW264.7 cells, even the concentration of 300 $\mu\text{g}/\text{ml}$ of MWCNTs [83]. In recent years, studies have shown that murine peritoneal macrophages exhibit impaired function when cultured with acid-functionalized single-walled carbon nanotubes (af-SWCNTs) due to their cytotoxic effects [84]. In the same laboratory, study also have showed that af-SWCNTs exhibit a cytotoxic effect depending on their concentrations [85].

Until now, two common toxicity screening assays have been used in scientific research, including in vitro and in vivo assays. These assays have been used in a growing body of evidence, and each method has both advantages and disadvantages involved in toxicological studies. In vitro assays provide information on the cellular mechanism of toxicity, while in vivo assays provide information at the organ level of toxicity of CNTs. For instance, in the early years, scientists studied the genotoxicity of aqueous colloidal solutions of multiwalled carbon nanotubes in in vivo experiments on laboratory mice. These results showed that MWCNTs can potentially affect the genetic material in mammals [86]. Most recently, two different types of carbon quantum dots (CQDs) prepared by ammonium citrate and spermidine, namely CQD-AC and CQDpd, were used to evaluate their biocompatibilities using the fish embryo acute toxicity test (FET). The results indicated that CQDs have no significant adverse effects and demonstrate high biocompatibility, owing to their low bioaccumulative properties in embryos, eleuthero-embryos, or adults, respectively [87]. Furthermore, a study related to the adverse effects of functionalized CNTs at the cellular level and their underlying toxicity-related mechanisms conducted experimental research both in vitro and in vivo models. These observations demonstrated differences in the toxicity under in vitro versus in vivo conditions [88]. In vivo assays have several disadvantages compared with in vitro assays, such as higher cost, complex data interpretation, greater biological variability, and being time consuming, etc.

In recent years, in vitro toxicity assessment methods been increasingly preferred in toxicologic research due to the advantages of lower cost, faster procedures, and fewer ethical concerns when compared with in vivo assays [89]. Uptake analysis, functional assays and viability assays are generally used as in vitro toxicity assessment methods. Uptake analysis is defined as the quantitative measurement and localization of cellular uptake. For instance, the cellular toxic effects of oxidized MWCNTs in the *Saccharomyces cerevisiae* experimental model were investigated, and the experimental results demonstrated that MWCNTs (> 100 mg/L) had toxic effects on the cell proliferation process. Researchers have investigated the cellular localization of MWCNTs. Finally, they elucidated the mechanism of MWCNTs uptake in the cellular process [90]. Functional assays are involved in the evaluation of the adverse effects of NPs on various cellular processes. For instance, in an earlier study, researchers constructed a hydrogel containing

SWCNTs for neural engineering applications and investigated the Schwann cell (SC) response to SWCNTs in both 2D and 3D microenvironments [91]. However, viability assays are only associated with screening the cell death induced by NPs exposure [92]. For instance, Liu et al. studied the adverse effects of carboxylated MWCNTs (MWCNTs-COOH) on the cellular apoptotic process and speculated that the modification of MWCNTs may reduce the toxicity of MWCNTs on L02 cells [93].

Due to the paucity of systematic studies investigating the exact mechanisms of CNTs cytotoxicity under both in vitro and in vivo conditions, most research has focused on speculating different mechanisms. Some studies have highlighted factors responsible for toxicity [89], including the physical properties of CNTs, preparation methods, characteristics of functionalized CNTs, and related parameters. For instance, Zijuan Qi and other researchers have conducted a comparative study of three kinds of surface chemically functionalized MWCNTs on lung cells and have reported that among the three groups, carboxylated MWCNTs (MWCNT-COOH) can facilitate mitophagy and cell-cycle arrest through acting as an enhancer of m6A methyltransferase METTL3/14 nuclear translocation, which in turn leads to m6A modification of mitophagy-associated genes, resulting in mitophagy-dependent cell cycle arrest, which is modulated by m6A methylation [94]. In a recent noteworthy study, researchers examined the effects of MWCNTs on three cell lines and demonstrated that MWCNTs not only facilitated intracellular drug delivery but also enhanced cytotoxicity by acting via a “Trojan horse” mechanism [95]. Another new study studied the genotoxic effect of SWCNTs using normal human astrocytes as a model and have revealed that even at low dose of SWCNTs such as 2 and 8 ng/mL of medium for 24 h toxic effect on the expression of several genes including DNAJB9, IGFBP3, IGFBP6, CLU, ZNF395, KRT18, GJA1, HILPDA and MEST mRNAs and some other miRNAs via 30-UTR binding sites. Scientists also speculated that endoplasmic reticulum stress induced by SWCNTs leads to disruption of gene expression [96]. Cytotoxicity properties of functionalized MWCNTs on pathogenic bacteria, such as *Pseudomonas aeruginosa* and *Staphylococcus aureus*, have also been studied in recent years [97]. Thus, it has become a mandatory task to rationally evaluate the toxicity of CNTs for a broad range of applications.

4.2 | Fullerene

The discovery of fullerene (C_{60}) was accompanied by intense scientific interest, as it greatly expanded the number of known allotropes of carbon [98, 99]. Due to its excellent physical and chemical properties, including resistance to heat, pressure, and radiation, as well as the high configurability of fullerene [100], it has become one of the most promising nanomaterials and has been widely used for materials science, nanotechnology, and in the pharmaceutical industry [101–104].

In addition to the wide range of applications of fullerene and its derivatives in different fields of science, their potential adverse effects at the cellular level have drawn the attention of researchers. For instance, in a series of experimental studies, researchers have revealed that the permeability and integrity of

cell membranes are disrupted by fullerene nanoparticles when they cross the membrane of the cell [105–109]. According to previous studies, scientists have also indicated that fullerenes not only have an adverse effect on protein activity, but also could affect protein function, including potassium channel proteins [110, 111], HIV proteases [112–116], serum albumins [117–119] and glutathione S-transferases [120–122]. A recent study has reported that fullerenes could bind to hydrophobic sites on the surfaces of proteins via π - π stacking interactions that exist between the conjugated surface of C_{60} and the hydrophobic residues of PHE, TRP, and TYR with aromatic rings [123, 124]. Despite these studies demonstrating the adverse effects of fullerene, there are several studies that have reported inconsistent results. For instance, in 2012, scientists reported cytotoxicity of five different complexes of C_{60} using Hep-2 cells as a model. The results showed that the sodium salt of the polycarboxylic derivative of fullerene C_{60} had a significant toxic effect against Hep-2 cells. While the other four types of complexes, including polyvinylpyrrolidone 10,000 and 25,000, C_{60} -NO₂ proline, and C_{60} showed no toxic effects on the same cell lines [125]. In a latest study, scientists investigated the toxic effects of a water-soluble fullerene derivative that produces fluorescence at the cellular level in vivo using *Drosophila melanogaster*. Researchers fed 40 μ g/mL fullerenes for 1 week and 1 month and investigated cytotoxic impacts on cell proliferation and fluorescence degeneration after entering the cell cytoplasm. After a series of quantitative and qualitative analyses, researchers demonstrated that fullerenes have cytoprotective effects and facilitate cell proliferation at the cellular level after they penetrate into the cell cytoplasm [126].

Even though numerous research studies have been conducted related to the cytotoxicity of fullerene and its derivatives, the detailed information regarding the atomistic interaction of fullerenes and the aforementioned proteins remains a “black hole” for the scientific community. Therefore, it is necessary to extensively study the cytotoxic effects of fullerene and its derivatives to support their myriad applications.

4.3 | Graphene and Its Derivatives

Graphene is an allotrope of carbon consisting of a single layer of atoms arranged in a two-dimensional honeycomb lattice, which was first obtained using the micromechanical exfoliation method in 2004 [127–131]. Graphene and its derivatives have a set of unique properties, such as a lattice nanostructure, high specific surface area, lightness, flexibility, and excellent mechanical integrity, which have placed them at the center of wide attention in many scientific fields. In general, graphene derivatives are mainly represented by two major forms, including graphene oxide (GO) and reduced-graphene oxide (rGO), where the former could be obtained by the oxidation of graphite and the latter could be synthesized by the reduction of GO. Graphene has not only shortened the gap between biology and electronics but also remarkably accelerated the development of a wide range of biomedical areas that can facilitate the integration of diagnostic, healthcare monitoring, and therapeutic modalities [132, 133].

The evaluation of in vitro cytotoxicity is critical to the potential widespread use of graphene-based nanomaterials in biomedical

applications. A series of studies have been performed to assess in vitro cytotoxic mechanisms of graphene-based nanomaterials. In earlier studies, researchers, for instance, indicated that the shape, exposure time, and concentration of graphene were critical factors in evaluating the degree of its cytotoxicity and cytotoxic mechanisms [134]. Vallabani et al. have studied the adverse effect of graphene oxide using normal human lung cells (BEAS-2B) within 24 h and 48 h of exposure at different concentrations, respectively. They demonstrated cell viability decrease in a dose- and time-dependent manner; however, the number of early and late apoptotic cells inversely increased, as determined by their MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay [135]. Through an experimental study with human MSCs derived from umbilical cord blood, Akhavan et al. confirmed that graphene's cytotoxic and genotoxic effects are dependent on both its dose and shape [136]. On the other hand, Lv et al. indicated that there was no cytotoxic effect of GO, but instead facilitated differentiation of SHSY5Y cells and improved expression of neuronal markers in an experiment with human neuroblastoma SH-SY5Y cells at a low concentration; they even suggested that GO can be a candidate for studying neurodegenerative diseases [137]. Some studies have also revealed that functional groups on the GO are also key factors in triggering toxicity. For instance, Das et al. reported the comparative study of GO sheets and reduced graphene oxide sheets (RGO), and found that GO was more toxic than RGO of the same size [138]. In a similar study, Teo et al. reported a cytotoxicity study on three types of graphene derivatives and concluded that graphene with a higher content of monofluoro-substituted carbon atoms was more toxic than the others [139]. In a recent study, the researchers conducted a comparative study on the cytotoxic mechanism of graphene oxide derivatives (GODs) in two types of mammalian cells, including normal cells (NIH3T3) and colon cancer cells (HCT116). They revealed that even though GOD-induced cytotoxicity (reduction of cell viability) in the two cell lines was not significantly different, the mechanisms of cytotoxicity triggered by GODs were substantially different. In HCT116 cells, DNA damage and cell death were induced by cell membrane leakage, whereas in NIH3T3 healthy cells, the main reasons for toxicity were oxidative stress and interaction between GODs and the cells [140]. This study revealed cell-dependent cytotoxicity of GODs.

4.4 | Nanodiamonds

Nanodiamonds (NDs) are characterized by carbon particles that have a diamond-like structure with a small size below 100 nm. It is speculated that NDs have more superiority in a wide range of scientific applications than other NMs, including drug delivery, imaging probes, coatings for implant materials, and biological applications [141] (Figure 7). However, the variety of biological applications of these emerging candidate carbon particles in science are hampered by their adverse impact on humans and other biological systems. The first scientific research related to the cytotoxicity of NDs was published in 2006 and demonstrated the non-toxicity of NDs ranging in size from 2 to 10 nm using different types of cell cultures [142]. Moreover, Yu et al. reported low toxic effects of NDs with a size of 100 nm when applied to kidney cell cultures [143].

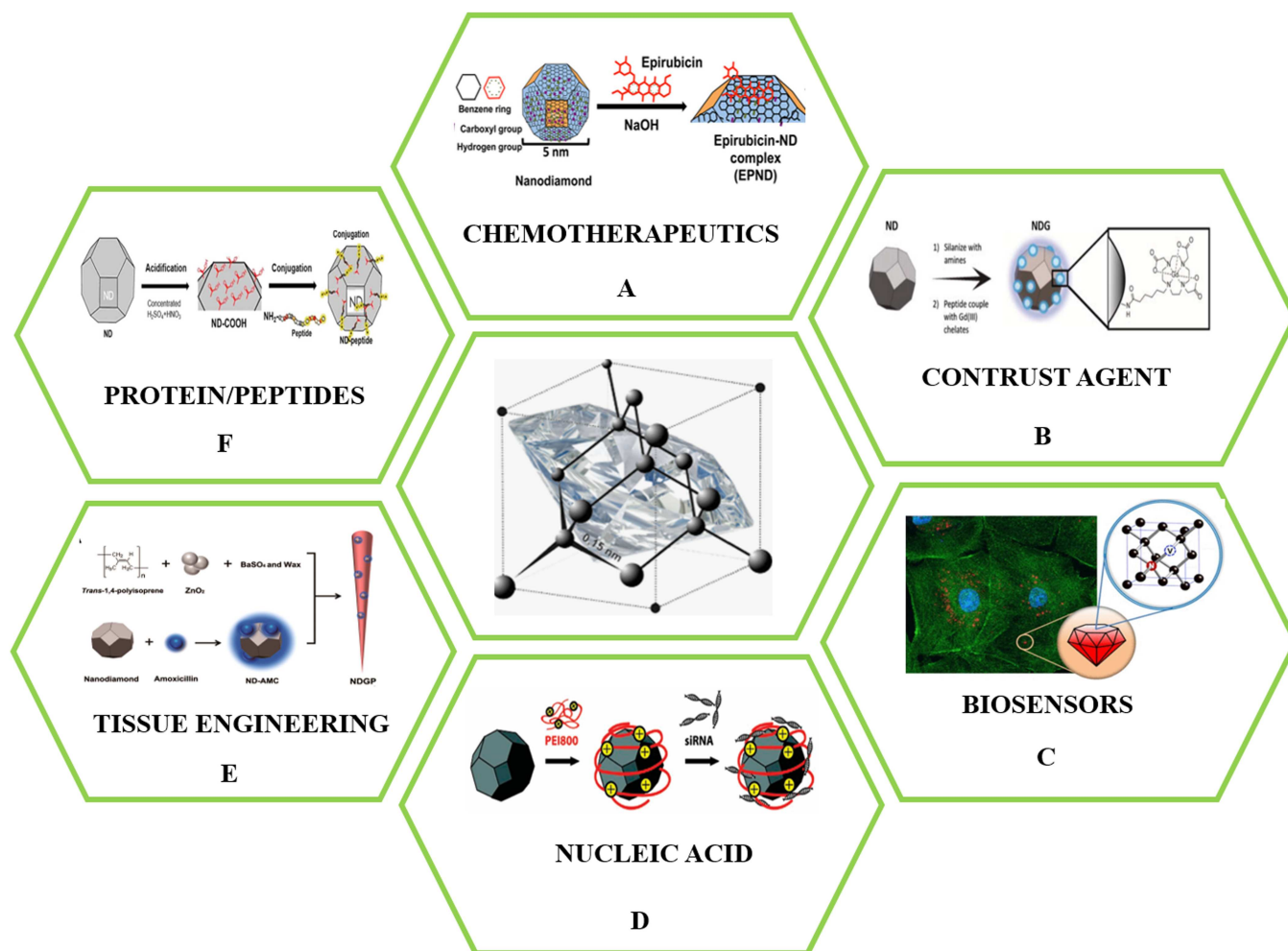


FIGURE 7 | Nanodiamond particle structure and applications in biomedicine adapted and remade from ref [141].

A recent study also demonstrated results consistent with earlier findings regarding the adverse effects of NDs [144]. In this study, researchers investigated the response of two types of NDs, including pristine and aminated NDs, to the formation and development of a protein corona, which consisted of bovine serum albumin and fibronectin. They found that both NDs triggered significant phagocytic cell death [145].

However, some studies have demonstrated significant cytotoxic effects of NDs compared to the aforementioned studies. For instance, researchers have evaluated the toxicity of four types of NDs particles using two types of cell line, including a human osteosarcoma cell line (MG-63) and primary rat mesenchymal stem cells (rMSCs). The results revealed that the toxicity of the four types of NDs was inversely proportional to their size [146].

In a latest study, scientists characterized two types of NDs and evaluated their adverse effects on mesenchymal stromal cells. NDs with a larger size exhibited lower toxic effects on the cells and were indicated to be ideal for bioimaging applications [147]. On a parallel track, a study has shown that the toxicity and sensitivity of two types of NDs to cells change according to oxidative stress triggered by atmospheric pressure cold plasmas (APCP) [148], and speculated that chemo-radiative stress may affect the cytotoxicity of NDs in exposed cells.

4.5 | Carbon-Based Quantum Dots

Carbon-based quantum dots (CQDs) were discovered by scientists accidentally in the early 2000s when conducting a study of SWCNTs. As an emerging member of the carbon-based nanomaterials family, they have been studied extensively for applications including fluorescent probes for in vivo bioimaging, drug tracing and imaging, in vitro diagnosis, cancer treatment, and many other biological realms [149–158]. For example, due to their photostability and fluorescent properties, CQDs exhibit up to 100-fold greater stability than most commonly used organic dyes, such as Rhodamine 6 G and Alexa Fluor 488 [159–166]. In a recent study, CQDs have been used as a diagnostic marker for cancer imaging and monitoring, and a novel system, an ultra-small copper sulfide QD-loaded diethyldithiocarbamate system, has been developed for cancer therapy [167, 168]. Despite extensive studies on CQDs, data on their adverse effects and underlying molecular mechanisms remain limited and sometimes contradictory. Despite extensive studies on CQDs, data on their adverse effects and underlying molecular mechanisms remain limited and sometimes contradictory. For instance, in a recent study, Yue et al. have developed the ruthenium-containing carbon dots (Ru-CDs) preparation platform and have evaluated the toxicity of Ru-CDs [157]. Finally, they revealed that Ru-CDs have no significant damage to the

cells but exhibit adverse toxicity through a concentration-dependent DNA photocleavage mechanism. The same results have been demonstrated one earlier study, have demonstrated that exposure of graphene quantum dots (GQDs) lead to proliferation of six type of cells [169]. Similar results were demonstrated in an earlier study, which showed that exposure to graphene quantum dots (GQDs) led to the proliferation of six types of cells.

On the other hand, in another study, researchers conducted a genotoxicity experiment on rat alveolar macrophages (NR8383), and e-demonstrated that after 24 h of exposure, amine-grafted graphene QDs triggered significant alterations in the expression of 2898 genes associated with the “cell cycle” [170]. In addition, a recent study demonstrated that even at a very low concentration of 0.025 ppm, CQD exposure caused severe damage to both human breast cancer cells (MCF7) and human primary dermal fibroblasts (HDFa) [171].

Furthermore, in another study, researchers have performed a systematic study on CQDs. In an in vitro study, scientists investigated the cytotoxicity of CQDs by using cell counting assay, the neutral red assay, lactic dehydrogenase assay, and fluorescein isothiocyanate (FITC) Annexin V/Propidium iodide (PI), as well as JC-10 assay methods, respectively, on the osteosarcoma 143B cell line. In addition, the adverse effects of CQDs were verified using Western blot and immunofluorescence analysis of apoptosis-related proteins. The results showed that CQDs exert a high level of toxicity towards cells and even led to the induction of cellular apoptosis. Further investigations through in vivo study observed antitumor activity of CQD by using animal models [172].

In conclusion, based on the above-mentioned literature, even though extensive data have been published related to CQDs, these ambiguous and incoincidence findings have hampered the broad range of biological and engineering applications of CQDs.

5 | In Vitro Toxicity of Carbon-Based Nanomaterials IN 3D Cell Culture

The advent of the 3D cell culture technique has been described as “Biology’s New Dimension,” highlighting its advantages such as being “near to cellular physiology” and “near-to-in vivo” [173]. This innovative strategy has seen exponential growth in research across various fields, including cancer biology, drug discovery, pharmacological applications, differentiation studies, tissue engineering, and gene and protein expression studies. Since the first paper on the subject was published in 1968, more than 1400 cell lines have been studied using the 3D cell culture technique by the year 2020 [174]. Despite the tremendous interest in CNTs within the scientific community, there are ongoing debates and ambiguous data regarding the toxicity of CNTs. These uncertainties have limited the wide-ranging applications of CNTs in scientific fields. Establishing standard protocols for examining the toxicity of CNTs in vitro models has become an urgent need to address this bottleneck in research. Generating standardized methodologies will contribute to a more comprehensive understanding of CNTs toxicity and

facilitate their safe and effective utilization in various scientific applications.

In the past decades, traditional applications such as 2D in vitro model system approaches have been employed for screening the toxicity of NPs. Even though this type of culture approaches have been used since the early 1900s [175, 176], this approach has several drawbacks in research. In recent years, 3D in vitro platforms, which more accurately replicate human physiology, have begun to replace traditional in vitro models. For instance, the first 3D tissue-mimetic models, established using an ultrasound standing wave trap to study the potential adverse cellular effects of SWNTs, were developed by scientists in early 2011. In this study, researchers have proceeded a comparison study of a conventional 2D cell model and a novel 3D cellular model, and have emphasized the priority of 3D tissue-physiological scenario models [177]. Another early study related to the adverse effects of carbon-based nanomaterials, three cell types of monocultures and triple3D cell co-cultures were studied for the investigation of oxidative stress and inflammation response in vitro of titanium dioxide and SWCNTs. The results have shown that all of the groups of cells have a tendency to triggering reactive oxygen species (ROS) except monocyte-derived dendritic cells (MDDCs). In addition, there was no significant tendency of increase or decrease in TAC (total antioxidant capacity) and the (pro-) inflammatory reactions in all four experimental groups after exposure to the NPs. Yet, in a group of advanced three-dimensional models of the human epithelial airways, TAC and IL-8 (Interleukin-8) had lower concentrations, whereas TNF- α had higher concentrations when compared to other cells. Finally, scientists concluded that 3D co-culture may be a better alternative to mimic the induction of cytotoxic effects of NPs in lung cell culture models [178]. However, researchers have investigated cytotoxic effects of multi-walled CNTs (MWCNTs) by using an Air-Liquid Interface Cell Exposure System (ALICE), combined with a 3D epithelial airway barrier model, which can mimic real inhalation. At the end of the study, they revealed that there was no significant cytotoxic induction in different exposure time slots [179].

In further studies, scientists have integrated diverse scientific research with 3D cell culture altogether for testing the toxicity of CNTs. For instance, a group of researchers investigated cytotoxicity and biodistribution pattern of three different types of carbon nanodots (CNDs) using a 3D lung epithelial tissue model. The results indicated that exposure to different types of CNDs for 24 h did not cause significant cytotoxic effects. Each type of CND also exhibited distinct physicochemical properties, which could influence their cellular [180]. Moreover, in another study, the adverse effects, such as inflammation and fibrosis of MWCNTs, were studied using 3D in vitro lung co-culture cell models when they were exposed to CNTs in different time periods. As a result, Scientists concluded that these CNTs may trigger a proinflammatory response, but not cytotoxicity or a significant profibrotic response in the cell models [181].

In the latest study, the adverse effects of mycotoxin sterigmatocystin (STE) were studied using in vitro 3D spheroid model from a human neuroblastoma cell line. When the spheroids were exposed to STE, the cells underwent

disaggregation in a time- and dose-dependent manner. Subsequent analysis revealed apoptosis induced by the exposure, as confirmed by immunofluorescence staining and Western blot. At the same time, STE cytostasis effects were investigated using migration assays in 2D and 3D cell culture, respectively. In the final step, the cellular adverse events closely related to the cytotoxicity of STE, such as ROS generation, as well as DNA damage, were observed. It is concluded from the above study that 3D spheroid model takes promising advantages over conventional methods in the study of cytotoxic effect of STE with the pivotal features of in vivo-like cell behavior [182].

A noteworthy study has recently developed 3D brain organoids derived from human induced pluripotent stem cells (iPSCs). This platform paves the way for scientists to investigate the human brain's behaviors in vitro. In the above-mentioned research, researchers have investigated the adverse effect of MWCNTs to 3D brain organoids that have expressed the brain cortical layer markers. In the end of the study, it has revealed that MWCNTs lead to induction of cytotoxicity to 3D brain organoids in a way of none- dose-dependent manner. In addition, mechanism of these cytotoxicities have been described, such as exposure to the MWCNTs could trigger a decrease in nNOS activity by inducing oxidative stress and regulating NF- κ B-KLF4 pathway [183]. Combined all of these results shed more light for toxicological study of the carbon-based nanomaterials in the realm of the science.

Conversely, by virtue of alluring chemical properties of nanomaterials such as CNTs, graphene, they are used for the detection of neurotransmitters so that can facilitate the adsorption of neurotransmitters and enhance electro-catalytic sensing in the intracellular environment of 3D cell culture. For instance, the latest study scientists have developed an integrated fluorescent sensors that can provide lively morphological and structural changes of the biological tissue by using mingled near-infrared fluorescent SWCNTs and Fmoc-diphenylalanine hydrogels [184]. Further study of other researchers have also demonstrated a novel imaging platform on the bases of fluorescent SWCNTs that can track thick brain tissue in the brain extracellular space (ECS), which contains 500 nm of post-synaptic densities in living samples. Finally, they have indicated a relationship the nanoenvironment and brain activity, depending on the diffusion parameters of ECS juxta-synaptic region that can be changed in response to neuronal brain activity [185].

In addition, in the hope of future designing 3D organs in vitro, scientists are passionate about the construction of dynamic scaffolds that incorporated CNTs into different types of scaffolds. For instance, the latest study has shown that titania nanotube scaffolds have been designed by scientists for neural implant applications using U87-MG glial cells and SH-SY5Y neuron cells [186]. In another study, researchers have demonstrated a one-step coating method for coating of CNTs in homogenously and non-toxic way to the surface 3D-printed scaffolds through make use of the electric-static force [187].

To the best of our knowledge, an organoid is defined as a miniature of organ that is produced by a 3D cell culturing technique in vitro [188]. In recent years, the drawbacks of

organoid cultures that have exciting potential in the realm of biomedical applications have been compensated by the integration of carbon-based nanomaterials with different 3D methods. For instance, recent studies have demonstrated the beneficial effect of CNTs with a concentration of 50 μ g/mL to the development process of intestinal organoids in between 0 and 5 days and have revealed the mechanisms that stimulate proliferation and differentiation of intestinal organoids. In addition, they have emphasized that the profitable effect of CNTs is closely associated with the layer of graphene in CNTs [189]. A set of in vitro investigations above allowed to clarify the mechanisms of carbon-based nanomaterials to biological cellular environment using innovated 3D cell culture technology.

6 | Toxicity Testing of Carbon-Based Nanomaterials: 2D Cell Culture versus 3D Conditions

The aim of toxicity testing carbon- based nanomaterials is eventually to be capable of speculate the possibility of an adverse event occurring in a target living organism. A novel milestone of transformation of toxicity testing from in vivo animal tests to in vitro toxicological tests have been announced by the US National Research Council in its report entitled Toxicity Testing in the 21st Century: A Vision and Strategy in 2007 [190, 191]. Within the superiority of lower cost for proceedings, reduced ethical consideration, cytotoxicological study in vitro play paramount important role in nanotoxicological study. However, unavoidable evidence is that CNTs toxicity-related issues provoke endless debates due to the inconsistency and controversy of data in a bulk of research, and even makes it urgent to construct a standardized toxicity assessment of CNTs, which hinders the large spectrum of applications of these alluring nanomaterials in the biological sciences.

Until now, even though the majority of the CNTs toxicity assays were conducted in 2D in vitro models, but by virtue of their physiological relevance, innovative and dynamic 3D cellular models have the potential to become a fundamental research tool in the realm of cytotoxicity. In addition, Organotypic 3D cell cultures will pave the way to meet the needs of both in vivo and in vitro studies. This is controversy tendency for both ethical concerns and economical reasons. In the same spirit, switching from 2D cultures to three-dimensional (3D) cultures is motivated by the need to create cellular models that better capture the complexities of cellular architecture. The 3D cell culture models present several advantages over the 2D cell culture models as follows: (1) More relevant in vitro systems for human studies, which is a vital challenge in toxicology; (2) Imitate a higher number of in vivo cellular features, whereas 2D have no potential to mimic the in vivo cellular environment. (3) In 3D culture can construct aggregate/spheroid structure closer to natural structure, while cells grow static, rigid in 2D; (3) 3D have capacity to replicate both cell-cell and cell-matrix interactions; (4) In 3D, cells will differentiate or proliferated depending on cell type, while in 2D, cells will proliferated with a high ratio and differentiate poorly. (5) Cell survival condition is closer to in vivo, such as the surface of spheroids consists of proliferating cells, where the interior possesses quiescent and necrotic cells; (6) Owing to high gene expression level [192]; the

toxicity of CNTs mostly relies on effects inside the cell chronically, even the expression of genes and tissue differentiation.

To sum up, 3D culture systems hold great promise for the vast majority of science in the long run. Although a plethora of experimental data regarding to adverse effect of CNTs, including in vivo, in vitro, and at the molecular level, have been published, displayed highly inconsistency in results. It is urgent to systematize and standardize the platform of CNTs toxicity and reveal the complexity of the mechanism of toxicity in detail. A quote “All models are wrong, some models are useful” –from a book of George E.P. Box [193], says each tool has its own superiority and thus own drawbacks [194, 195]. Despite the numerous advantages, 3D models are not sufficient for advancing our understanding of potential adverse effects associated with CNTs. Augmentation of more innovative techniques will be necessary to make the most of the benefits of using 3D models in toxicity research.

7 | Challenges of Strategies in Assessment of Nanotoxicity of Carbon Nanomaterials in 3D Cell Culture

An immense interest in regard to a plethora of applications of CNTs due to its tempting properties has been facilitated the vast production, widely usage of different types of CNTs. However, the environmental and human exposure to CNTs is still a major concern. A variety of regulations associated to safety and toxicity of the CNTs have been constructed in different countries. For instance, precise exposure limits of the CNTs have been pinpointed in 2013 by US National Institute for Occupational Safety and Health [196]. In the same spirit, CNTs have been placed to SIN (“Substitute It Now”) list as the first nanomaterials in recent years after the evaluation of carcinogenous effect of MWCNTs on to human health by the Swedish non-profit organization ChemSec [197].

Despite of there are a bulk of research data concerning about the toxicity of CNTs at molecular, cellular, and in vivo levels, the results are almostly contrivorsial as we all know. There are some reasons for these conflicting results. Firstly, the risk assessment of CNTs is most tough task due to the difference of CNTs from conventional compounds in many aspects, such as their unique physical, chemical, electrical, and mechanical properties. Secondly, the behavior of CNTs in cellular conditions, including deposition on cells, permeation of acellular barriers, cellular uptake, and change by the exposure conditions are more complex than conventional compounds.

Until now, a number of different approaches, such as epidemiology studies, human clinical studies, animal studies, and in vitro studies, have been used for the assessment of adverse effect of CNTs. With the priority of lower cost, faster, and minimum ethical concerns, in vitro CNTs risk assessment is an important method amongst above mentioned methods. Especially, the advent of novel 3D cell culture models had improved the in vitro cell culture technique thoroughly. Now, both monolayer 2D cell culture and 3D cell culture model have been used widely for the assessment of cytotoxicity of CNTs. Even various comparison studies reported higher cytotoxicity of NPs in 2D than in 3D culture [198–209] (Table 1).

In vitro cell culture encounters a bottleneck in accurately imitating in vivo physiological conditions. Although various 3D models have been developed to better mimic the real cellular environment, there are challenges in adapting cell viability assays, primarily constructed for monolayer 2D cell culture, to the 3D cell culture systems [210]. Likewise, toxicological research on carbon-based nanomaterials reveals limitations in representing the real in vivo cellular environment. Investigating the adverse effects of carbon-based nanomaterials on cells over the long term poses challenges due to the unexpected consequences of chronic simulation. For instance, studies on the acute and long-term effects of industrially produced MWCNTs on human lung and immune cells showed that acute exposure to high doses of MWCNTs resulted in high levels of ROS and decreased metabolic activity, with large amounts of nanotubes accumulating in A549 cells. However, no major adverse long-term effects were observed in 6 months of exposure. Researchers also pointed out that MWCNTs did not induce adaptive mechanisms against particle stress in long-term treated A549 cells, while previous studies had revealed that cells developed protective mechanisms against particle-induced stress [211, 212]. Another challenging aspect of nanomaterial toxicity, including carbon-based nanomaterials, is the undefined dose of CNTs in vitro assays, impeding the ability to ascertain true toxic potentials. Despite numerous studies on the concentration-dependent toxicity of CNTs, there is a paucity of a reasonable explanation to elucidate the relationship between CNTs and exposed cell lines. Hurdling these obstacles will contribute to more accurate and comprehensive assessments of the toxicity of carbon-based nanomaterials.

8 | Concluding Remarks and Future Roadmaps

CNTs, owing to alluring physical and chemical properties, CNTs have emerged as highly promising materials across diverse scientific fields, including drug delivery and gene therapy, cancer research, tissue engineering, immunology, electronics, and energy applications. Despite this broad applicability, these innovated materials remain widely restricted in usage due to insufficient understanding of their cytotoxic and long-term biological effects.

First and foremost, Nanotoxicology—a subdiscipline of toxicology, has garnered attention in the scientific community over the last two decades [213–215]. This field focuses on studying the potential toxic interactions between nanomaterials and living organisms, including cells and tissues. Early nanotoxicity studies of CNTs relied predominantly on in vivo animal models and conventional 2D monolayer cell cultures. While these approaches have provided valuable initial insights, they remain fundamentally inadequate for deciphering complex toxicity mechanisms at the cellular and tissue levels. Flat and rigid 2D fail to recapitulate native cell-cell and cell-extracellular matrix interactions, whereas in vivo models are often limited by high-cost, low-throughput, ethical constraints, interspecies variability, and limited mechanistic resolution. Collectively, these drawbacks highlight an urgent need for advanced and standardized in vitro platforms capable of more predictive nanotoxicity assessment.

TABLE 1 | Cytotoxic Comparison Studies of CNTs in 2D Cell Culture and 3D Cell Culture.

Type of CNTs	Cytotoxic test	Type of cells or cell line	Year	Comparative cytotoxicity studies	Reference
SWNTs	AlamarBlue assay	PC12 and 3T3	2008		[198]
SWNTs	High-content screening and analysis (HCSA)	THP-1 cells	2011	Cytokine (IL-6 and TNF- α) secretion levels were measured using HCSA after exposure to p- and o-SWNTs for 24 h. The results revealed that there was no any toxic effect of the 3D cellular model in a 24 h exposure to CNTs. While 2D cell cultures cytokine (IL-6 and TNF- α) secretion levels were significantly increased by exposure to p- and o-SWNTs after 24 h. From experimental results it is concluded that 3D cell culture was provided more tissue-mimetic manner and cost-effective solutions for toxicity screening assays.	[199]
MWCNTs	ROS, enzyme linked immunosorbant assay (ELISA), Cell viability	MDM	2012		[202]
Quantum dots	[3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] (MTT), glucose-6-phosphate dehydrogenase (G6DP), and calcein AM and ethidium homodimer (calcein AM/EthD-1)]	HeLa cells and osteosarcoma MG63 cells	2012		[203]
MWCNTs	Formazan bioreduction (MTS) assay	EAhy926 cells	2013		[204]
SWCNTs and MWCNTs	Oxidative stress; risk assessment	MDM, MDDC, 16HBE14o, sophisticated triple cell	2014		[205]
MWCNTs	ROS, Lactate dehydrogenase (LDH) release assay	Human (alveolar) epithelial A549 cell line, combined with human monocyte-derived dendritic cells (MDDCs) and macrophages (MDMs)	2015		[179]
MWNCNTs	ROS; palmitic acid.	HUVEC and A549 monocultures	2016		[206]
MWNCNTs	Cell viability	HBMECs	2017	In the study, cytotoxicity of MWCNTs and acid oxidized MWNTs with or without a phospholipid-polyethylene glycol coating were investigated in both 2D and 3D cell culture respectively. In the end of the research scientists have	[207]

(Continues)

TABLE 1 | (Continued)

Type of CNTs	Cytotoxic test	Type of cells or cell line	Year	Comparative cytotoxicity studies	Reference
MWNTs	Autophagy; ER stress	Human umbilical vein endothelial cells (HUVECs)	2018	advocated that 3D cell culture is more predictive than 2D in toxicological study.	[208]
Nanodiamond particle	ROS, Real-time cytotoxicity assay (RTCA)	Rat hepatoma cells (Fao)	2019		[209]
CNTs	Cell viability	Human vocal fold fibroblasts	2019		[200]
SWNTs	Sensor responses to progesterone	Mice lines	2023		[201]

In this context, 3D cell culture technologies represent a critical methodological shift rather than a mere incremental improvement. Platforms such as SpAi (A Machine-learning Supported Experimental Workflow for High-throughput Spheroid Production and Analysis) [216] spheroids, organoids, and scaffold-based systems offer enhanced physiological relevance by better mimicking tissue architecture, biochemical gradients, and dynamic cell–matrix communication. Importantly, these systems enable improved assessment of chronic and low-dose CNTs exposure, conditions that are poorly captured by both 2D cultures and short-term in vivo assays. While 3D platforms have not yet fully replaced traditional models, their continued refinement strongly suggests a future in which they become the standard framework for nanotoxicity testing.

Despite these advances, significant challenges remain unresolved. Long-term exposure triggered unpredictable chronic simulation, undefined variability in the dosage, etc., are some challenging issues in the research field. Moreover, variability in 3D construct size, composition, and maturation state complicates inter-study comparisons and regulatory translation. Addressing these limitations is essential for the next phase of progress in CNTs nanotoxicology. Looking forward, the evolution of 3D nanotoxicity platforms will depend on their integration with complementary and emerging technologies. Furthermore, a key trend in future research is the integration of conventional in vitro models with 4D cell culture technologies in CNTs nanotoxicology. In particular, the fourth dimension in cell culture refers to the incorporation of time, enabling dynamic changes in cell structure, behavior, and the cellular microenvironment. This approach holds great promise for next-generation nanotoxicity assessment of the time-dependent, long-term CNTs exposure.

The convergence of 3D cell culture with artificial intelligence-driven data analysis, advanced biomaterial engineering, microphysiological systems, and bioreactor-based dynamic culture platforms offers a powerful roadmap for the field. Such hybrid systems hold the potential to enable real-time monitoring of cellular responses, improved dose–response modeling, and mechanistic insight into CNTs-induced toxicity pathways. Future research directions include promising areas, such as microfluidic organ-on-a-chip platforms, smart biomaterials, multi-omics approaches, and advanced imaging techniques, which may facilitate more accurate and reliable predictions of the adverse effects of CNTs. Ultimately, these advances will support the development of more predictive, reproducible, and human-relevant nanotoxicity assessment frameworks, accelerating the safe and responsible translation of carbon-based nanomaterials.

Author Contributions

Ayimgül Uzunyol: conceptualization, methodology, writing – review and editing, writing – original draft, visualization, resources, data curation. **Fatih Inci:** validation, funding acquisition, writing – review and editing, supervision, data curation.

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Ethics Statement

The authors have nothing to report.

Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

The authors have nothing to report.

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