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In vitro toxicity of carbon nanotubes: a systematic review†

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Carbon nanotube (CNT) toxicity-related issues provoke many debates in the scientific community. The controversial and disputable data about toxicity doses, proposed hazard effects, and human health concerns significantly restrict CNT applications in biomedical studies, laboratory practices, and industry, creating a barrier for mankind in the way of understanding how exactly the material behaves in contact with living systems. Raising the toxicity question again, many research groups conclude low toxicity of the material and its potential safeness at some doses for contact with biological systems. To get new momentum for researchers working on the intersection of the biological field and nanomaterials, *i.e.*, CNT materials, we systematically reviewed existing studies with *in vitro* toxicological data to propose exact doses that yield toxic effects, summarize studied cell types for a more thorough comparison, the impact of incubation time, and applied toxicity tests. Using several criteria and different scientific databases, we identified and analyzed nearly 200 original publications forming a "golden core" of the field to propose safe doses of the material based on a statistical analysis of retrieved data. We also differentiated the impact of various forms of CNTs: on a substrate and in the form of dispersion because in both cases, some studies demonstrated good biocompatibility of CNTs. We revealed that CNTs located on a substrate had negligible impact, *i.e.*, 90% of studies report good viability and cell behavior similar to control, therefore CNTs could be considered as a prospective conductive substrate for cell cultivation. In the case of dispersions, our analysis revealed mean values of dose/incubation time to be 4–5 μg mL^{-1} h^{-1}, which suggested the material to be a suitable candidate for further studies to get a more in-depth understanding of its properties in biointerfaces and offer CNTs as a promising platform for fundamental studies in targeted drug delivery, chemotherapy, tissue engineering, biosensing fields, *etc*. We hope that the present systematic review will shed light on the current knowledge about CNT toxicity, indicate "dark" spots and offer possible directions for the subsequent studies based on the demonstrated here tabulated and statistical data of doses, cell models, toxicity tests, viability, *etc*.

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Introduction

Carbon nanotubes (CNTs) attracted the tremendous interest of the scientific community because of their diverse applications in electronics, photonics, composite materials, and as part of energy sources and storage systems.[1–3] Landmark papers published by Iijima,[4,5] where the structure of multiwalled carbon nanotubes (MWCNTs) and single-walled carbon nanotubes (SWCNTs) was visualized, ignited a great scope of R&D activities. A remarkable combination of physical and chemical properties, intensively studied in the next few years, pushed researchers' interest towards integrating CNTs into biosystems. Therefore, CNTs were also proposed for biomedical applications such as tissue engineering and regeneration, target drug delivery, hyperthermia treatment for selective cancer cell killing, gene therapy, bioimaging, biosensing, as electrodes for neural prosthetics, *etc.*[6–11] Such great attention of researchers was driven by a unique alliance of nanoscale size and exceptional mechanical, optical and electrical characteristics that make CNTs attractive for a direct contact with living systems.[12,13]

In 2000, scientists, for the first time, successfully combined the new material with the most sensitive living system, neurons, giving momentum to the relatively innovative cross-disciplinary field – nanotechnology for biomedical tasks.[14] However, in the following years, a large number of publications also demonstrated a negative impact of CNTs on biosystems related to hydrophobicity of CNTs, low synthesis-to-synthesis reproducibility of the material characteristics and their unclear acute toxic and long-term biological impacts.[15–26] On the contrary, some studies demonstrated non-toxic effects or apparent toxicity of CNTs in contact with biological systems.[27–33] Metal catalyst impurities, CNT structure and geometry all stemming

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from the synthesis method, surfactants and functional groups can greatly affect the final CNT toxicity decision.[24] Although the Materials Science and Engineering community continues to explore practical applications of the material, toxicologists are reasonably concerned with dangerous consequences and substantial negative impact of the CNTs on human health.

Nowadays, the scientific community has identified three possible mechanisms of cellular CNT toxicity. The first one is based on irreparable mechanical injury of membrane (cellular or nuclear).[24] With high probability, the endocytosis, phagocytosis, or nano penetration, which are the main ways of the nanomaterial interaction with a lipid membrane, strictly depends on CNT geometry, especially length.[21,34] The next proposed toxic mechanism is oxidative stress occurring because of the increased level of reactive oxygen species (ROS)[24] and leading to numerous side effects in the cell such as apoptosis, necrosis, cytochrome c release, oxidative DNA damage, proliferation reduction, inhibition of cells growth, G2/M cycle arrest, *etc*. The final mechanism, genotoxicity mechanism, is somehow related to DNA damages characterized by a broad spectrum: the interaction of CNTs with proteins participating in chromosomes aberration, the impact of CNT on the mitotic spindle, micronuclei formation, indirect DNA oxidation, DNA breakage, *etc.* Despite the fact that the toxic mechanisms of CNTs are studied from several points of view, there is still a strong dependence of triggered or inhibited molecular pathways and cell types. A lot of the well-known signaling cascades are involved in different cell responses to materials, several of which are investigated in-depth in case of CNT impact: MAPK, AP-1, NF-κB, Akt, NLRP3 inflammasome, TGF-β1, and p53.[24,35] Despite the described complexity of occurring processes inside the cells that were targeted by CNTs, some research papers propose ways to overcome the CNT toxicity impact by modification the material surface with functionalization groups,[36] coating with metal oxides[37] or with proteins attachment.[38] For example, coating with recombinant C1q, which is a protein activating classic pathway of the complement system involved in innate immune system, is a perspective approach of the inflammation regulation.[39] Besides, several theoretical studies, devoted to modelling of possible cellular response to CNTs, demonstrate a nanotubes mechanical interaction with a lipid layer[40–42] or with proteins,[43,44] proposing the safer CNT geometry, which deepen the understanding of CNT actions on cells.

In recent years a significant increase in CNT industrial production for electronics such as touch screens, composites fabrication, and other applications resulted in huge concerns about industrial workers' health hazards, environmental impact, and related needs of the development of the standardized protocols for safety guidance.[45–51] In some publications, researchers have already highlighted that the main entry ways of nanomaterials into the human organism are mouth, nose, or skin, directing to the digestive tract, respiratory system or resulting in skin erosion, respectively (Fig. 1).[52,53] Although the described exposure scenarios may have negative impact on human health, which is already traditionally comparable with asbestos because of the quite similar form, it is still essential to get more in-depth information about the toxicity mechanisms on a base cellular level (*in vitro*). This might help to reveal ways to overcome toxicity limits by the nanomaterial functionalization or surface modifications.[7,54] Thus, role of CNT physical parameters (type of CNT, length, diameter, synthesis method, catalysts, *etc.*) towards substantial biological effects has been addressed in several reviews,[7,15,21,55–59] along with cell type, toxic dose, mechanisms of toxicity and other essential parameters[24,60] in attempt to make classification to reveal common trends.[15,21,61] However, the numerous data related to substantial biological effects such as apoptosis, life cycle arrest, reactive oxygen species (ROS) production, and gene expression give only vague toxicity prognosis and uncertain identification of toxic doses. In addition, some original works hint at untouched parameters or characteristics of CNTs to be acknowledged. For example, Sweeney *et al.* demonstrated that carboxylated MWCNTs show reduced toxicity towards macrophages,[62] but the work of Dong *et al.* demonstrated the fully *vice versa* effect for the same cell type.[63] The difference between studies relies on MWCNTs produced by different companies (CheapTubes Inc. or Chengdu Organic Chemicals Co. Ltd), used cell viability assays (MTS assay or CellTiter-Glo test), types of macrophages (human or mice), and as a result culture medium (RPMI or DMEM). Likely, because of these reasons, according to the literature, the toxicity of CNT is located in the very broad range from 5 ng mL^{-1} up to 10 mg mL^{-1}, which differs six orders of magnitude.[21]

At the same time, one more critical aspect that should be undoubtedly considered in the context of the material toxicity assessment and environmental impact is the so-called "bioaccumulation" phenomenon of material. The process is defined as the absorption of a chemical by a living organism through all possible routes happening in the natural environment.[64] The recent review of Bjorkland *et al.*, where 42 original references were collected to perform a potential examination of CNTs bioaccumulation in different species, revealed that trophic transfer of CNTs, or food chain, is negligible and absorption of CNTs through epithelial barriers is also low.[65]

According to the latest standards, every new drug or material proposed to somehow affect the human organism should be carefully tested for acute toxicity or long-term perspectives.

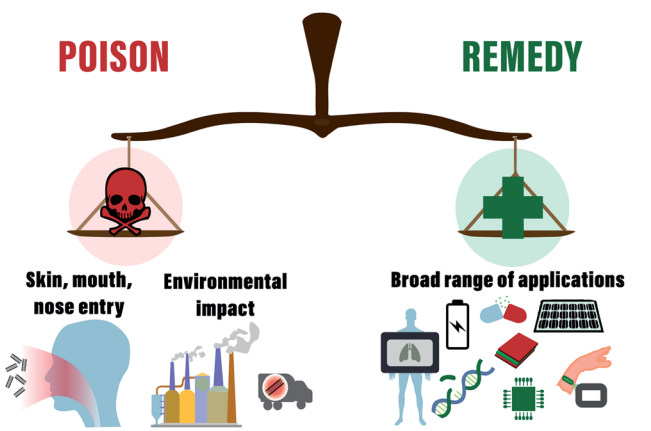

Fig. 1 Schematic presentation of CNTs applications and their threat to environment and humans.

Toxicology, historically established as a science of poisons,[66] nowadays is a multidisciplinary field covering studies of all known synthetic and natural substances with a goal to test toxic effects and identify safety level.[67] According to P. Paracelsus, well-known "Father of Toxicology," all substances are poisons and the right dose differentiates a poison from a remedy.[67] To verify chemical toxicity and identify safe doses, several guidelines were developed by European Centre for the Validation of Alternative Methods, Interagency Coordinating Committee on the Validation of Alternative Methods and Organization for Economic Cooperation and Development (OECD) for standardized methodology of laboratory practice for new compounds or drug testing. In particular, the ISO 10993-protocol formulated for "Biological evaluation of medical devices" in 2009 includes rigorously precise steps for toxicity evaluation. Depending on contact time, location in the organism, authors prescribe various tests for evaluation of cell morphology, cytotoxicity, genotoxicity, inflammation and other parameters (Fig. 1). As a result of the researchers' concern for applying nanomaterials in biosystems, nanotoxicology was also accepted as a distinct discipline in 2017.[68] To study safeness or hazard effects of materials at the nanoscale, new protocols were recently formulated by the Food and Drug Administration (FDA) (ISO/TR 10993-22:2017).

Looking at the current examinations of the CNT toxicity for *in vitro* models in the frame of this mini-review, we have realized the absence of the prescribed protocols among the studies. This fact could be explained by the need to consider many characteristics, starting from the type of CNTs, type of contact with cells (on a substrate or in the form of dispersion) to long-term and high costs of these tests. Still, several publications demonstrated quite promising results of OECD prescribed tests employing CNTs synthesized by OCSiAl (Russia), by Bayer Technology Service GmbH (Germany), by Hanwha Nanotech Corp. (Korea), and by the European Commission Joint Research Centre (JRC, Italy).[69–72] Consequently, every research group follows its own way of "toxicity evaluation" with various cell lines, cytotoxicity tests, incubation time, doses, *etc*. Therefore, the scientific community faces an extraordinary situation when many experiments to study CNT toxicity were conducted, but the straight doses causing toxic effects are still unclear, data about biological effects vary greatly and the toxicity question is scarcely addressed.

Hereby, we perceived the importance of competent assessment of the material's possible toxic effects to answer the question: "is the devil as black as he is painted?" We systematically evaluated studies performed *in vitro* and traced possible correlations between the type of CNTs (SWCNT or MWCNT), the way of contact with cells (substrate or dispersion), cell type, incubation time, dose and cytotoxic test that greatly influence the decision about the CNT toxicity. We also realized the need for a new approach of literature analysis in this field, so the present mini-review is a systematic collection of articles which establishes common trends regarding the CNT toxicity. The present mini-review focuses only on the results of *in vitro* toxicity studies following the PRISMA statements, a set of elements aimed to help authors how to perform and report systematic reviews,[73] broadly used in the biomedical field (the methodology and search strategy are described in the next chapter in more details).[74–76]

The central part of the present work is divided into three chapters. Each chapter discusses in detail one of the critical parameters applied for original references classification. The first part examines CNT synthesis procedure to identify companies where materials were tested *in vitro* and industrial impact on the scientific field of CNT toxicology in time perspective. The second and the third parts summarize data about CNT applied in the form of substrate or dispersion, respectively. In addition, for these two chapters, we collected data about cell type, incubation time, the used design of CNT, synthesis procedure, applied cytotoxic tests, and dose/viability parameters. Thus, based on the collected data and references to original articles, one would be able to get the needed information quickly and orient in the broad field of CNT toxicity.

Methodology of search strategy

The databases MEDLINE, Cochrane Database of Systematic Reviews, PROSPERO were searched to guarantee this systematic review would not duplicate any works. Scopus and Web of Science databases were studied for the relevant publications; extraction date is 12 April 2021. Articles were independently searched by two authors (M. C. and F. F.).

Criteria formulation

We used SPIDER formulation: sample (S), phenomenon of interest (PI), design (D), evaluation (E), research type (R), to identify our search strategy. Thus, we focus on *in vitro* CNT toxicity studies on mammalian cells with positive or negative outcomes and with quantitative or qualitative assessment.

The results of studies with animals, *in vivo*, are less comparable than those ones *in vitro* using cells. This is because of numerous factors: different species (mice, rat, rabbit, guinea pig, *etc*.), way of drug administration (oral, intraperitoneal, intravenous, *etc*.), observation time (several hours, days or months), dose range and many others parameters that should be constantly controlled such as food supply, gender, light, *etc*. In contrast, conditions for cell studies are precisely guided: temperature, humidity, percent of CO_2, nutrients supply, *etc*. Besides the more convenient use in laboratories, *in vitro* models could be applied for proposing effects and prediction for *in vivo* studies.[77–79] Based on these facts, we have chosen the *in vitro* model for collecting existing literature data and our analysis.

Criteria for exclusion and inclusion

We excluded reviews, conference abstracts, opinions, commentaries, review book chapters from the search results. We included only original articles containing data with qualitative or quantitative assessments of cell viability.

Requests for databases

For the Scopus database we used the next request: KEY ((“carbon nanotube*” OR swcnt* OR mwcnt* OR cnt*) AND

(toxicity OR toxicology OR biocompatibility OR toxic OR cytotoxicity OR genotoxicity OR genotoxicology OR nanotoxicology OR nanotoxicity) AND ("cell interaction*" OR "*in vitro*" OR cell*) AND NOT (animal*)) AND (LIMIT-TO (DOCTYPE, "ar")) AND (LIMIT-TO (LANGUAGE, "English")). For the Web of Science: AK=(("carbon nanotube*" OR swcnt* OR mwcnt* OR cnt*) AND (toxicity OR toxicology OR biocompatibility OR toxic OR cytotoxicity OR genotoxicity OR genotoxicology OR nanotoxicology OR nanotoxicity) AND ("cell interaction*" OR "*in vitro*" OR cell*)).

Data extraction

In the review, we collected the information about types of CNT, official distributors or manufacturers (companies), way of contact with cells (substrate or dispersion), type of cells, incubation time and type of tests for toxicity analysis.

Results and discussion

In the Scopus database, we found 1124 articles that fulfilled our request; in the Web of Science database, the same request resulted in 196 articles. The reference search helped us to reveal 38 related publications. After filtering the publications, sorting, and lists combination, we obtained 194 articles with the required data forming the central core of the papers in the field of CNTs *in vitro* toxicity.

CNT synthesis

The CNT origin can be globally attributed to two groups: produced by a company or synthesized in a research laboratory. Commercially produced tubes were found to be more widespread among researchers for testing of the CNT toxicity, *i.e.*, in about 60% of the found publications CNTs are purchased from companies. The most popular CNTs for cytotoxic studies were produced by Carbon Nanotechnologies Inc. (USA), Cheap Tubes Inc. (USA), Nanostructured and Amorphous Material (Nano-Amor) Inc. (USA), Shenzhen Nanotech Port Co. Ltd (China), Sigma-Aldrich Inc. (USA) and Nanjing XFNANO Materials Tech Co. Ltd (China) as summarized in Table S1, ESI.† There are two largest centers serving as sources of commercial CNTs utilized in toxicology studies (Fig. 2A). The first one is located in the USA and the second one in Asia. While researchers from different disciplines address the toxicity of CNTs: engineering, biology, medicine, materials science, many laboratories are not related to the field of materials science, *i.e.*, they do not synthesize the material. Therefore, the about the synthesis method, catalysts, and geometry of CNTs is often limited by the data provided by the manufacturer.[80–82]

In Fig. 2B, we present the dynamics of appearance of the CNT toxicological studies for the time period, starting from the first publications in 2000 until 2021. The first pioneer works included data of cell cultivation directly on thin films made of CNTs.[14,83–85] The results of these studies were quite promising because cell viability and morphology were similar to the control group kept without contact with CNTs. Intrigued by these first experiments, researchers brought greater attention to the topic, yielding the growth in publications in 2005–2007. Herein, researchers also studied the effects of CNTs on living cells in a dispersion related to possible applications in biology and medicine as drug carriers. The 2005–2007 period is characterized by an expected increase of CNTs industrial production driven by the developed CNTs synthesis technology at the macroscale and increased industry demands.[36] For example, Nano Carbon Technologies Co. Ltd (Japan) and Shenzhen Nano Technologies Port Co. Ltd (China) grew quickly and were already producing 5 kg of CNTs per hour by 2007.[86] Nowadays, worldwide CNT demand is reported to be more than 2000 tons per year for aerospace industry, composite production and battery manufacturing with market growth up to USD 9.84 billion by 2023.[87] In many companies, such as Showa Denko, OCSiAl, Hanwha Nanotech, the annual volume of production is already greater than 100 tons.[88]

Starting from 2007, the scientists' interest in the CNT bioeffects was fully switched to CNTs in a form of dispersion facilitated by the increased industrial production and humanity fears about the toxic effect on the environment and on employees involved in the synthesis of the materials.[89] Several years later, it was evident that being easily absorbed onto the skin surface, CNTs internalized through epithelial tissues forming barriers in a human organism (Fig. 1).[15,46,90] The time was crucial for further applications of CNTs in the industry of composite materials, plastic, rubbers and biomedicine. From 2010, most publications were focused on the effect of CNTs in dispersions, and the interest in CNTs as a substrate for cell growth was reduced.

According to our analysis, MWCNTs were identified to be the most popular material in cytotoxicity studies (Fig. 2D). That fact can be explained by wider availability of the material and the proposed safer nature of MWCNTs because of their greater diameter, decreasing the chances of cell membrane damage and tube penetration into cells.[18] Numerous studies proposed a toxic impact of SWCNTs in dispersions because of possible penetration into cells, while MWCNTs have an indirect impact by facilitating changes in cell microenvironment.[18,23]

Summing up, scientists' attention to the toxic effects of CNTs is driven by the massive industrial growth of application in energy storage systems, sensors, composites and transparent microsized electronics.[91,92] Nearly 60% of all tubes employed in cytotoxicity tests are of industrial origin where the USA and Asia represent locations with the highest impact. Thus, MWCNTs were in more demand likely because of the availability.

Tested cells types

Fig. 3A and Table S2 (ESI†) summarizes all cell types tested in the collected articles published between 2000 and 2021. Nearly 80 different types of cells were tested by researchers for cytotoxic effects of CNTs. A large part of these studies was done using the regular A549 cell line, human alveolar epithelial cells.[93] This cell line is widely used and recommended as a convenient model for toxicological studies.

Human Umbilical Vein Endothelial Cells (HUVECs) is the second most common type of cells, usually used to study blood

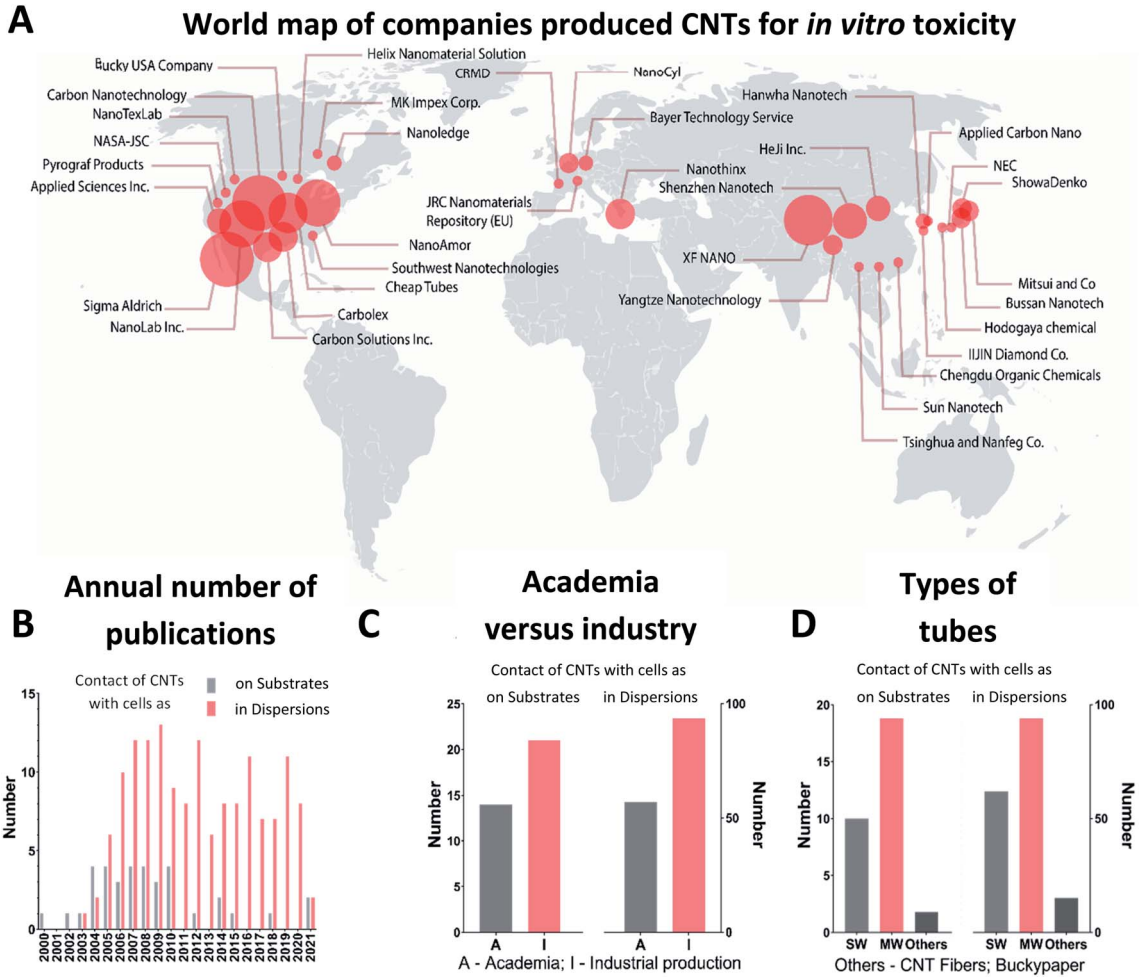

Fig. 2 Production of CNTs employed for *in vitro* assessment of toxic effects – comparing industry and research laboratories, *i.e.*, academia. (A) Companies that produced CNTs applied for *in vitro* toxicity assessment and their world location with two centers: the USA and Asia. (B) The annual number of cytotoxic studies devoted to direct contact through dispersion or substrate included in the statistical analysis in our review. (C) Number of papers where CNTs synthesized by industry (I) or by research laboratories, *i.e.*, academia (A). (D) Types of tubes used in studies: SWCNTs (SW), MWCNTs (MW) or others (CNT fibers or Buckypaper).

vessel regulation.[94] The next one is BEAS2B epithelial line from a human lung that is also suitable for toxicity testing.[95] Close to BEAS2B were other lung epithelial cell types and less than 3% of studies employed osteoblasts, skin fibroblasts, hippocampal neurons and several other cell types.

Thus, to verify the effects of pristine or functionalized CNTs, to check the impact of the synthesis approaches, catalysts, and

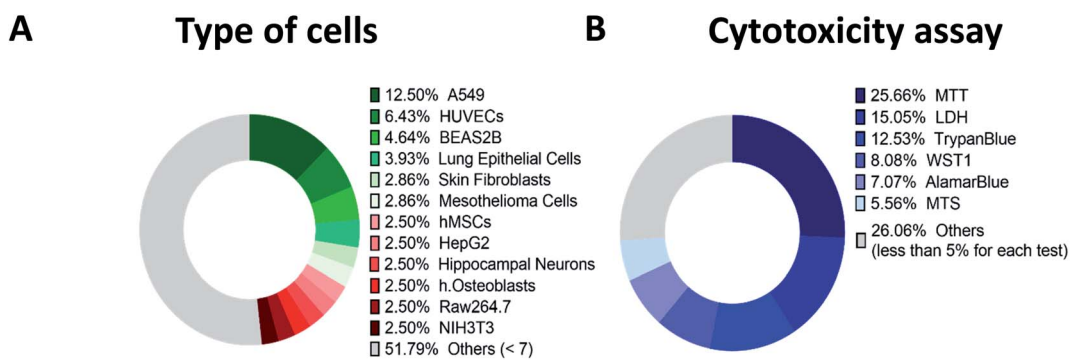

Fig. 3 Statistics for two crucial parameters greatly varying among *in vitro* examinations: cell types and applied cytotoxic test. (A) Cell types used in the studies included in the present review analysis; (B) tests used for evaluation of the CNT toxicity in dispersion.

CNT geometry, we identified the most employed models - A549, HUVECs or BEAS2B cells.

Impact of CNTs applied on substrates

As we mentioned earlier, one of the parameters influencing cytotoxic effects is the way the tubes contact with the cells – serving a substrate or in a dispersion. First studies included data of cell incubation on substrates covered with CNTs.[14,83–85] We summarized these 36 publications in Table 1.

Among the listed publications, we found only several reports about the toxic effects of CNTs on substrates on cell physiology, meaning that CNTs play a role of a harmless surface for cell growth.[84,96,97]

The listed studies in Table 1 can be divided into four main groups according to the used cells types: cells related to nervous system,[14,96,98–109] osteoblasts,[81,83,84,96,110–112] fibroblasts,[84,96,107,111,113–119] and other types.[84,97,119–127]

In the case of neurons, glia or cortical cultures growth, each study demonstrated a successful outcome. Thus, in 2000 the first attempts were made to study the interface between CNTs and living systems. Mattson *et al.* successfully applied CNTs as a substrate for growth of embryonic rat hippocampal neurons.[14] The researchers used MWCNTs (diameter of 20 nm, length of 20–100 μm) prepared with the catalytic decomposition of a ferrocene-xylene precursor, dispersed in ethanol and applied to cultural plastic as substrates. Moreover, they covered some samples with 4-hydroxynonenal (4-HNE) to study the effects of modified tubes on neurite outgrowth.[129] The molecule 4-HNE plays the role of a "crossroads" substance with numerous functions in cells, *e.g.* regulating gene expression, the proliferation of cells, cell death, and stress-mediated pathways.[130] The scientists successfully demonstrated neuron adhesion, neurite outgrowth, and branching of cells grown on modified substrates. This first work opened the door connecting two fields, nanotube technology and neurobiology, which inspired many researchers to move further and study the interaction of the material with cells in more detail.

The other study was conducted with dissociated cortical cultures on a substrate patterned with CNTs "clusters".[98] After several days of incubation, researchers found that cells were located directly on the CNT clusters and formed interconnections, what demonstrated successful engineering of the neural network and its evolution.

In 2004, the research group tested the effect of chemically modified CNTs onto isolated hippocampal neurons.[99] The researchers revealed that a pristine, or as-prepared (AP), SWCNT film was a permissive substrate; better branching of cells was observed onto the tubes modified with ethylenediamine (EN) (MWCNTs-EN). Based on these experiments, a vital conclusion followed - surface charge impacts the neurite outgrowth. In the same year, Webster *et al.*[96] evaluated the possible application of CNTs as neural implants. The researchers revealed that the addition of CNTs in the composite resulted in inhibition of glial cells, which allowed to control glial to neuron cell ratio and glial scar formation. Increased postsynaptic activity of neurons grown onto CNTs[101] and modulation of cell morphology[102] was demonstrated for these cell cultures. Electrical stimulation through the modified substrate covered with CNTs enabled control over cell growth and cells differentiation.[100,103–105,107–109,129] A good material biocompatibility with cells was also confirmed by Dubin *et al.*[106] In the case of osteoblasts, researchers showed an enhanced proliferation[83] and higher growth rates onto CNTs when compared with regular implant materials like Ti_6Al_4V or CoCrMo,[84] and concluded the material to be promising for orthopedic applications.[81,110–112]

Fibroblast cultures were shown to have the same viability as in the control group,[113,115–119] long-term growth without abnormalities in nuclei and regular morphology,[106,107,114] so the material was also proposed to be promising for tissue restoration.

According to our analysis, the number of publications devoted to toxicity of CNTs placed onto substrates is four times lower when compared to works studying CNT effects in dispersion. The authors of the present review propose that such a shift could be due to the high rate of methods development to disperse CNTs and their possible impact in dispersion on the environment and human health by means of internalization through epithelial tissues.[89]

Furthermore, CNTs dispersed in liquids have wider application range than those placed onto the substrates or just in the form of thin films because of greater probabilities for contact with cell membranes and internalization into cells. For instance, CNTs are rather promising as a substrate for orthopedic or implant application, tissue engineering, and as electrodes or conductive substrates for electrically active tissues. At the same time, CNTs in the dispersion may be applied for target drug delivery, photonics, biosensing, bioimaging, gene therapy, *etc*.

Impact of CNTs in dispersion applied to cell cultures

To find some correlations and obtain a clear picture of the field, the authors of the present review made several assumptions. Firstly, we divided all cells into four groups according to tissue types – epithelial, connective, muscle, and nervous. Secondly, we considered a dose and incubation time accounting for the type of the CNTs only, *i.e*., disregarding the extra parameters such as functionalization or tube modifications, tube purification procedure and synthesis. Also, we roughly assessed the toxicity of materials as a factor influencing cell viability. However, in reality, the material may affect cell metabolism, gene expression and other aspects of cell physiology. Authors understand that such assumptions lead to significant simplification, but it seemed to be the only chance to identify some core trends of the CNT toxicity in dispersion *in vitro*.

The most significant part of *in vitro* in dispersion studies was done with the commonly used MTT colorimetric assay (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2*H*-tetrazolium bromide) for cell viability identification; tetrazolium dye MTT is reduced to formazan with a purple color whose intensity correlates with a number of alive cells; near 26% of all utilized test types are MTT tests (Fig. 3B). However, in several works, researchers demonstrated that MTT is sometimes unreliable because of the

Table 1 Cytotoxic studies of CNT thin-films substrates for cells

Type of cells or cell line	Incub. time (hours (h) or days (d))	Type of CNTs, diameter (D), length (L)	Synthesis, producer, catalysts, functionalization (F)	Year	Ref.
Embryonic rat hippocampal neurons	8 d	MWCNTs, $D = 20$ nm, $L = 20$–100 μm	Catalytic decomposition, ferrocene-xylene, 4-HNE (F)	2000	14
Osteoblasts	3, 7 d	MWCNTs, $D = 60$–200 nm	CVD, Applied Sciences Inc., USA, pyrolytic aromatic hydrocarbon layer (F)	2002	83
Human osteoblasts, ovine bladder smooth muscle cell, mouse skin fibroblasts, human articular chondrocytes	1 h	MWCNTs, $D = 60$–200 nm	CVD, Applied Sciences Inc., USA, pyrolytic aromatic hydrocarbon layer (F) (composite with polycarbonate urethane)	2003	84
Dissociated cortical cultures from one-day-old Charles River rats	Near 6 d (150 h)	No data about tube parameters	Clusters of CNT, iron nitrate as the catalyst	2004	98
Fibroblast mouse L929	1, 7 d	MWCNTs (vertically aligned), $L = 50$–35 μm	CVD, Ni catalyst, NanoLab Inc., USA	2004	113
Hippocampal neurons from 0 to 2 day Sprague-Dawley rats	7 d	No data about tube parameters	AP-SWCNT, MWCNT-COOH (F), MWCNT with ethylene diamine(F), poly-*m*-aminobenzene, CVD	2004	99
Rat astrocyte cells, pheochromocytoma cells, human osteoblast cells (embryo 3T3) mouse fibroblasts	1 h, 3 d	MWCNTs, $D = 60$ nm	CVD, Applied Sciences Inc., USA, pyrolytic aromatic hydrocarbon layer (F)	2004	96
Hippocampal neurons	8, 10 d	MWCNTs, no data about tube parameters	Nanostructured and Amorphous Material (NanoAmor) Inc., USA	2005	101
Hippocampal neurons	3, 7 d	SWCNTs, no data about CNT parameters	SWCNT-COOH (F), Carbon Solutions Inc., USA	2005	102
H19-7 (derived from hippocampi from embryonic day 17 Holtzman rats)	6 h	MWCNTs (vertically aligned), $L = 10$ μm	CVD, iron catalyst	2005	128
NG108-15 neuroblastoma × glioma hybrid cells	3, 5, 10 d	SWCNTs, no data about tube parameters	Arc-discharge, CarboLex Inc., USA	2005	108
NG108-15 neuroblastoma × glioma hybrid cells	3, 5, 10 d	SWCNTs, no data about CNT parameters	Arc-discharge, CarboLex Inc., USA	2006	109
Human osteoblasts hFOB, human fibroblastic line HS-5	24, 48 h, 7 d	MWCNTs, no data about tube parameters	Catalytic decomposition on CoO/MgO catalyst	2006	111
Osteoblasts ROS 17/2.8 cells	5 d	SWCNTs, $D = 1.5$ nm, length varied, MWCNTs, $D = 10$–30 nm	SWCNT-COOH(F); SWCNT-PABS(F); SWCNT-PEG(F); Carbon Solutions Inc., USA	2006	81
Mouse embryonic neural stem cells	3, 5, 7 d	SWCNTs, no data about tube parameters	HiPCO, Carbon Nanotechnologies Inc., USA	2007	104
Hippocampal cultures from 0/3 d old Sprague Dawley rats	8, 14 d	SWCNTs, no data about tube parameters	HiPCO, Carbon Nanotechnologies Inc., USA	2007	103
Human skin fibroblasts, Schwann cells from rats, cortical and cerebellar neurons, dorsal root ganglion neurons	36–48 h, 14 d	MWCNTs, $D = 10$ nm, $L = 300$ μm	CVD	2007	107
Osteoblast	21 d	MWCNTs, no data about tube parameters	CVD, cobaltous nitrate solution	2007	112
Immortal NIH3T3, primary, rat hippocampal neural cells	14, 24 h, 7 d	SWCNTs, no data about tube parameters	Nanoledge Inc., Canada	2008	106
Fibroblast L929 mouse cells	2, 24, 48, 72, 96 h	MWCNTs, no data about tube parameters	—	2008	116
Human osteoblast cells	7 d	MWCNTs; $D = 10$–20 nm, $L = 1$–20 μm	CVD, NanoLab Inc., USA	2008	110

Table 1 (*Contd.*)

Type of cells or cell line	Incub. time (hours (h) or days (d))	Type of CNTs, diameter (D), length (L)	Synthesis, producer, catalysts, functionalization (F)	Year	Ref.
Human collateral cancer cell Caco-2, human breast adenocarcinoma MCF7 and HL-60, primary HA-SMCs	24, 72, 120 h	—	CVD, NanoLab Inc., USA	2008	97
Hippocampal neurons of newborn Sprague-Dawley rats	3 d	SWCNTs, no data about tube parameters	Carbon Solutions Inc., USA	2009	105
Neural stem cells	12, 24 h, 7 d	SWCNTs, no data about tube parameters	HiPCO, Carbon Nanotechnologies Inc., USA	2009	129
Human embryonic stem cells	1, 3, 5 d	—	—	2009	121
NIH-3T3	24, 48 h	MWNTs $D = 5$–15 nm	CVD, NanoLab Inc., USA	2010	115
Human mesenchymal stem cell	1, 3, 7, 14 d	SWCNTs, no data about tube parameters	Carbon Solutions Inc., USA	2010	122
Human embryonic stem cells	1, 7 d	MWCNTs, no data about tube parameters	Sigma-Aldrich Inc., USA	2010	124
Mouse fibroblast cells	24, 48, 72, 96 h	MWCNTs, no data about tube parameters	Microwave plasma chamber, Ni or Fe layer	2010	117
Mouse fibroblast cells	6, 48, 72 h, 7 d	—	Microwave plasma chamber, Ni or Fe layer	2012	118
Mouse embryonic fibroblasts, human bronchial epithelial cell line 24 h	24 h	MWCNTs, $D = 6$–15 nm	—	2014	119
Human HCC lines (SNU182 and HUH7)	3 d	Vertically aligned CNTs, no data about CNT parameters	CVD	2014	125
Human promyelocytic leukemia cell line HL-60, human 134 histiocytic lymphoma cell line U-937, the human chronic leukemia cell line K-562	24, 48, 72 h	—	CVD, NanoLab Inc., USA	2015	123
Pancreatic adenocarcinoma, PANC-1, AsPC-1, and BxPC-3 cell lines	1, 4, 8 d	MWCNTs, forest, no data about tube parameters	CVD	2018	126
Murine L929 fibroblasts, human dermal fibroblast	1, 3, 7 d	MWCNTs, no data about tube parameters	Nanostructured and Amorphous Material (NanoAmor) Inc., USA	2021	114
HEK-293 cells, neonatal rat ventricular myocytes (NRVMs), murine Bone marrow derived macrophages, Jurkat cells, SH-SY5Y	2, 5 d	CNTF, $D = 22.2 \pm 0.7$ μm	—	2021	127

Table 2 Cytotoxic studies of CNTs applied as dispersion

Type	Cytotoxic test	Incub. time	Dose, viable (*V*)/dead (*D*) cell	Cell type	Year	Ref.
SWCNT	Alamar blue	18 h	0.24 mg mL^{-1} *D* 37.6%	Human epidermal keratinocytes	2003	149
SWCNT; MWCNT	Alamar blue	24, 48, 72, 144 h	144 h 0.16 mg mL^{-1} *V* 50%	hMSCs	2008	135
SWCNT (solution-indirect)	Alamar blue	24, 48, 72 h, 96 h	Indirect 24 h 0.8 mg mL^{-1} *V* 90%; direct 24 h 0.4 mg mL^{-1} *V* 60%	A549	2008	150
MWCNT	Alamar blue	24 h	BEAS2B IC_{50} = 7 μg mL; mesothelioma IC_{50} = 17 μg mL^{-1}	BEAS2B; mesothelioma	2012	151
MWCNT	Alamar blue	24 h	50 μg mL^{-1}; *V* 70%	BEAS2B	2013	82
MWCNT	Alamar blue	24 h	Different data (1 μg mL^{-1} is safe; other conc varying data)	BEAS2B; MESO1	2014	152
MWCNT	Alamar blue	24 h	10 mg L^{-1}, *V* > 60%	Primary astrocytes	2015	145
SWCNT; MWCNT	Alamar blue	24, 48 h	(SW)100 μg mL^{-1}; *V* near 90% for both times; (MW) EC_{50} = 10 μg mL^{-1}	THP1	2017	153
MWCNT	Alamar blue	24 h	24 μg mL^{-1}; *V* > 50%	BEAS2B; SAEC	2019	154
MWCNT	Alamar blue	24 h	100 μg mL^{-1}; *V* > 80%	Human lymph node endothelial cells	2020	155
SWCNT	Alamar blue (for substrates contact NIH3T3 and PC12)	15 min RBC, 3 d	-;- (RBC lysis); *V* for NIH3T3; PC12 as in control	RBC; NIH3T3; PC12	2008	120
MWCNT	Alamar blue, LDH	24 h	BEAS2B 10 μg mL^{-1} *V* 0%; IMR32 100 μg mL^{-1} *V* 70%; THP1 10 μg mL^{-1} *V* > 60%	BEAS2B; mesothelioma; neuroblastoma (IMR32), THP1	2012	156
SWCNT; MWCNT	Alamar blue, MTT	24 h	A549 0.75 mg mL^{-1} *V* 100%; U937 varied data	A549; U937	2007	138
SWCNT	Alamar blue, NR, MTT	24 h	EC_{50} = 744 ± 91 μg mL^{-1}; with serum > 800 μg mL^{-1}	A549	2007	157
SWCNT	Annexin V + PI	4 h–24 h	10–50 μg mL^{-1}, no cells activation	Primary immune cells	2006	158
SWCNT	Annexin V + PI	24, 48 h, 72, 96 h	50 μg mL^{-1} *V* 100% for all time points	A549	2007	159
SWCNT; MWCNT	Annexin V + PI	24, 48 h, 72 h	48 h EC_{50} = 450 μg mL^{-1} (SW); 72 h EC_{50} = 600 μg mL^{-1} (MW)	Mononuclear cells	2012	144
MWCNT	Aqueous one	24, 96 h	BEAS2B 4 d 200 μg mL^{-1} *V* near 20%; A549 4 d 200 μg mL^{-1} *V* 80%	BEAS2B; A549	2011	160
MWCNT	CCK8	24 h	50 mg mL^{-1} *V* > 60% (N); 50 mg mL^{-1} HeLa *V* 80%	Neuroblastoma (N); HeLa	2012	161
MWCNTs	CCK8	24 h	40 μg mL^{-1} *V* 50%	HepG2	2016	162
MWCNT	CCK8	24, 48 h	100 μg mL^{-1} *V* > 80% for both times	NIH-3T3	2016	163
MWCNT	CCK8	72 h (+26 weeks)	For 72 h 1.92 μg cm^{-2} *V* near 100%	HBEC-3KT	2017	164
MWCNT	CCK8	6 h	64 μg mL^{-1} V near 60%	HUVECs	2018	165
SWCNT	CCK8	12 h	50 μg mL^{-1} *V* near 50%	HUVECs	2018	166
SWCNT	CCK8	12 h	50 μg mL^{-1} *V* near 60%	HUVECs	2018	167
MWCNT	CCK8	24 h	60 μg mL^{-1} *V* near 80%	HUVECs	2019	168
MWCNT	CCK8	24 h	64 μg mL^{-1} *V* > 80%	THP-1	2019	169
MWCNT	CCK8	24 h	64 μg mL^{-1} *V* > 60%	HUVECs	2019	170
MWCNT	CCK8	24 h	64 μg mL^{-1} *V* > 60%	SMCs smooth muscle cells	2019	171
MWCNT	CCK8	24 h	64 μg mL^{-1} *V* > 60%	HUVEC	2019	172
SWCNT	CellTiter blue assay	6 h–48 h	-;- (factor increase units)	A549; NHBE	2008	173
MWCNT	CellTiter blue	24, 72 h	24 h 200 μg mL^{-1}, *V* > 80%	Raw264.7	2012	174
MWCNT	CellTiter-Glo	24 h	25 μg mL^{-1} *V* > 80%	Human brain microvasculature endothelial cells	2017	175
SWCNT	CellTiter-Glo	72 h		HEK293; MCF10A; MRC-5; HepG2	2017	176

Table 2 (*Contd.*)

Type	Cytotoxic test	Incub. time	Dose, viable (*V*)/dead (*D*) cell	Cell type	Year	Ref.
			10 μg mL^{-1} *V* > 60% for the most cases (only one type decreased to 10%)			
SWCNT	CyQUANT	24 h	25 μg mL^{-1} *V* 100%	Human lung fibroblasts; HUVEC	2013	177
SWCNT; MWCNT	Flow cytometry	24, 48, 72 h	24 h 100 μg mL^{-1} *V* 90%; 72 h 100 μg mL^{-1} *V* 80%	Jurkat	2008	139
MWCNT	Flow cytometry	8, 24, 48 h	Jurkat S 100 μg mL^{-1} *V* 100%	Jurkat; primary splenocytes (S); primary neurons (N)	2009	178
MWCNT	Flow cytometry	1 h	100 μg mL^{-1} *V* near 50%	A549	2015	179
MWCNT	Flow cytometry	24 h	200 μg mL^{-1} V near 50%	Normal liver cells	2015	180
MWCNT	Flow cytometry	24 h	120 μg mL^{-1} *V* near 50%	Raw264.7	2020	181
SWCNT	Hemocytometer (cells recovery)	Up to 4 d	50 μg mL^{-1} – (30% or 85% from control)	Murine lung epithelial cells	2007	80
MWCNT	Hoechst 33342 + microscope	24, 48 h	0.6 μg mL^{-1} *V* 60% for 48 h	Human skin fibroblasts	2005	182
MWCNT	Indirect assay (protein expression)	24, 48 h	—	HEK keratinocytes	2006	183
MWCNT	Indirect assay (AP detection kit)	2, 4 h	—	Mouse embryonic Stem cell	2007	184
SWCNT	Indirect assay (NO, free radicals, super oxide)	1–2 h	0.5 mg mL^{-1} no changes	Raw 264.7	2005	185
SWCNT	Indirect assay (NO, free radicals, super oxide, apoptosis)	24, 48, 72 h	30 mg mL^{-1} *D* 10% for 48 h	J774; hMDM	2006	186
SWCNT	Indirect assay (oxidative stress)	3 h	10 μg mL^{-1} ROS increase in 12%	BJ Foreskin cells	2007	187
MWCNT	LDH	24 h	125 ng mL^{-1} no toxic effects	Human mononuclear cells	2007	188
MWCNT	LDH	24 h	—	hMSCs	2013	189
MWCNT; SWCNT	LDH	24 h	40 μg mL^{-1} *V* 100%	MDMs	2014	190
MWCNT; SWCNT	LDH	24 h	100 μg mL^{-1} *V* 50% for both	A549; J774	2014	141
MWCNT	LDH	24 h	80 μg mL^{-1} *V* 80%	Met-5A	2016	191
SWCNT	LDH	24, 48 h	2 μg mL^{-1} *V* 50% for both times	HeLa; HUVEC; Hep2G	2018	192
MWCNT	LDH	24 h	—	Mesothelial LP9	2020	193
MWCNT	LDH, trypan blue	24 h	12 μg mL^{-1} *V* > 60%	Bronchial epithelial primary cells	2019	194
MWCNT	LDH, CCK8	24 h	100 μg mL^{-1} *V* 50%	HUVEC	2014	195
MWCNT	LDH, MTT	48 h	300 ng mL^{-1} *V* < 40%	HEK293	2010	196
MWCNT	LDH, MTT, WST1, XTT	1, 72 h	48 h LDH 100 mg mL^{-1} *V* 60%; XTT 100 mg mL^{-1} *V* 80%	A549	2008	197
MWCNT	LDH, trypan blue	24 h	TC_{50} = 20–80 μg mL^{-1} (for both CNT)	A549; Hep3B; HEK	2010	198
MWCNT	LDH, WST1	24 h	EC_{50} near 200 μg mL^{-1}	A549	2017	199
MWCNT; SWCNT	LDH, WST1	24 h	256 μg mL^{-1} *V* 100% (MW); (SW) 64 μg mL^{-1} *V* 100%	16HBE	2018	143
MWCNT	LDH; WST8	24 h	32 μg mL^{-1} *V* near 80%	HUVECs	2017	200
MWCNT	Live/Dead	12, 24 h	5 μg mL^{-1} *V* 30%	Rat LE cells	2010	201
SWCNT	Microscopy	6 d	—	Primary neurons (N); primary glia (G)	2009	202
MWCNT	Microscopy	48 h	17 μg mL^{-1} *V* 20%	DRG; PC12	2010	203
MWCNT	Molecular probes cell viability	24 h	5.5–55.8 μg mL^{-1} *V* > 50%	Human osteoblasts	2004	85
SWCNT	MTS	24, 48, 72 h	0.04 mg mL^{-1} *V* 100% for all time points	MCF7; human epidermal keratino-s	2004	204
SWCNT	MTS	24, 48, 72 h	40 μg mL^{-1} *V* 90%	MCF7	2015	205
MWCNT	MTS	24 h	100 μg mL^{-1} *V* for all > 50%	A549; lung V79 fibroblasts	2015	206
MWCNT	MTS	24 h	400 μg mL^{-1} *V* > 50%	Raw264.7	2016	207
MWCNT	MTS	24 h	IC_{50} 10 μg mL^{-1}	A549	2021	208
SWCNT	MTS, flow.cyt.	24 h	40 μg mL^{-1} *V* 100%	MCF7	2013	209

Table 2 (*Contd.*)

Type	Cytotoxic test	Incub. time	Dose, viable (*V*)/dead (*D*) cell	Cell type	Year	Ref.
MWCNT	MTS, LDH	24 h	100 μg mL^{-1} *V* 100%	BEAS2B; RLE6TN; THP1	2013	210
MWCNT; SWCNT	MTS, live/dead	24 h	(MW) 10 μg cm^{-2} *V* 90%; (SW) 10 μg cm^{-2} *V* 100%	HAEC	2012	136
SWCNT	MTS, NR, LDH	24 h	100 μg mL^{-1} resulted in first cytotoxic ef (varied EC_{50} 233 μg mL^{-1} to 600 μg mL^{-1})	Caco2	2009	211
SWCNT	MTT	72 h	20 μg mL^{-1} *V* 20%	Human epidermal keratino-s; HeLa; A549; H1299	2005	212
SWCNT; MWCNT	MTT	3 h, 6 h	22.6 μg cm^{-2} *V* > 80% for 6 h (MWCNT); *V* 40% (SWCNT)	Alveolar macrophages	2005	142
SWCNT	MTT	24 h–120 h	100 μg mL^{-1} *V* 50%	HEK293	2005	213
SWCNT	MTT	24, 48 h	20 μg mL^{-1} *D* 80%	Human dermal fibroblasts	2006	36
SWCNT; MWCNT	MTT	24 h–120 h	25 μg mL^{-1} *V* > 80% for both	Human dermis fibroblasts	2006	134
MWCNT	MTT	24, 96 h	0.2 μg mL^{-1} H596 24 h *V* > 60%; H466 24 h *V* > 20%; calu 24 h *V* > 20%	H596; H466; Calu-1	2006	214
SWCNT	MTT	3 d	30 μg mL^{-1} *V* < 50%	Mesothelioma MSTO-211H	2007	215
SWCNT; MWCNT	MTT	24 h	100 μg mL^{-1} (N) *V* 80% for MW; *V* 40% for SW; (M) *V* 40% for MW; *V* 20% for SW	Neuroblastoma (N); Rat alveolar macrophages (M)	2007	216
SWCNT	MTT	6 h, 48 h	500 mg L^{-1}; Hep3B and HepG2 *V* 80%; for Panc-1 40%	Hep3B; HepG2; Panc-1	2007	217
SWCNT	MTT	24, 48, 72 h	24, 48 h 3.2 μg mL^{-1} *V* 90%; 72 h 3.2 μg mL^{-1} *V* 80%	Astrocytoma 1321N1	2008	218
MWCNT	MTT	24 h	100 μg mL^{-1} *V* 50%	HeLa	2009	219
MWCNT	MTT	24 h	17.5 μg mL^{-1} *V* 90%	Primary cortical cultures	2009	220
MWCNT	MTT	24, 48 h	24 h D384 100 μg mL^{-1} *V* 25%; A549 100 μg mL^{-1} *V* 50% (same for 48 h)	A549; astrocytoma D384	2010	221
MWCNT; SWCNT	MTT	48, 72 h (up to 3 w for diff)	24 h and 48 h 30 μg mL^{-1}, *V* > 60% (for both CNT)	MSCs	2010	137
MWCNT	MTT	72, 144 h	30 μg mL^{-1} *D* near 15%	A549; Jurkat	2011	222
SWCNT; MWCNT	MTT	24, 72, 168 h	(SW) 0.11 mg mL^{-1} *V* near 100% up to 168 h; (MW) 0.11 mg mL^{-1} *V* 50%	A549	2011	223
MWCNT	MTT	24 h	100 μg mL^{-1} *V* 50% for both lines	A549; Raw264.7	2011	224
MWCNT	MTT	24, 48, 72 h	40 μg mL^{-1} *V* 90%; 200 μg mL^{-1} *V* 90%; 400 μg mL^{-1} *V* 80%	NHDF	2012	225
MWCNT	MTT	24 h	100 μg mL^{-1} *V* 60%	A549	2012	226
MWCNT	MTT	24 h	6 mg mL^{-1} for one type *V* 40%, for another near 20%	J774	2012	227
MWCNT	MTT	12, 24, 36 h	24 h 100 μg mL^{-1} *V* 80%; 200 μg mL^{-1} *V* 70%	C6 rat glioma	2012	228
MWCNT	MTT	24 h	A549 TC_{50} = 35.6 μg mL^{-1}; HepG2 TC_{50} = 33.5 μg mL^{-1}; HEK TC_{50} = 35 μg mL^{-1}; P407 TC_{50} = 39 μg mL^{-1}	A549; HepG2; HEK; P407	2014	229
SWCNT	MTT	24 h	50 μg mL^{-1} *V* 100%	A549; MCF7; SKBr3	2015	230
MWCNT	MTT	24 h	2 μg mL^{-1}, *V* (L) near 50%; *V* (S) near 70%	A549	2016	231
MWCNT	MTT	48 h	200 μg mL^{-1} *V* 40%	HT29	2017	232
MWCNT	MTT	48 h	256 μg mL^{-1} *V* 50%	A549	2019	233
MWCNT; SWCNT	MTT	48 h	8 μg mL^{-1} *V* > 80%	A549	2020	234

Table 2 (*Contd.*)

Type	Cytotoxic test	Incub. time	Dose, viable (*V*)/dead (*D*) cell	Cell type	Year	Ref.
SWCNT	MTT	24 h	200 μg mL^{-1} *V* > 80%	A549	2020	235
MWCNT	MTT, LDH	24 h	40 μg mL^{-1} *V* 60%	A549	2012	236
SWCNT	MTT, LDH	48 h	IC_{50} = 87.6 μg mL^{-1}	HEK293	2014	237
MWCNT	MTT, LDH	24 h	100 μg mL^{-1} *V* 70% (TT1); *V* 70% (ATII), AM *V* 55%	Alveolar type-I like cells (TT1), ATII, alveolar macrophages	2014	238
MWCNT	MTT, LDH	1, 3 h	—	A549	2020	239
MWCNT	MTT, NR	24 h	EC_{80} = 2.1 mg L^{-1}	H295R (adenocarcinoma); T47Dluc	2014	240
SWCNT	MTT, LDH, WST1	24 h	50 μg mL^{-1} MTT *V* 60%; LDH *V* 100%; WST1 *V* 100%	A549; HUVEC; alveolar macrophage	2006	133
MWCNT	MTT, WST1	72, 96 h, 1–2 w	3d 5 μg mL^{-1} *V* > 80%; 1 w 5 μg mL^{-1} *V* 80%	SH-SY5Y	2009	241
MWCNT; SWCNT	MTT, LDH	24, 48 h	24 h (SW) 300 μg mL^{-1} *V* 65%; 24 h (MW) 300 μg mL^{-1} *V* 65%; 48 h (SW) 150 μg mL^{-1} *V* 40%; 48 h (MW) 150 μg mL^{-1} V 50%	NIH3T3	2009	242
SWCNT	MTT, LDH	24, 48, 72 h	50 μg mL^{-1} *V* 60% (for both tests)	HUVEC	2010	243
SWCNT	MTT, LDH	24 h	100 μg mL^{-1} *V* 20%	PC12	2010	244
SWCNT	MTT, LDH; trypan blue	24 h	50 μg mL^{-1} V > 80%	Human umbilical cord MSCs	2020	245
SWCNT	MTT, WST	24 h	MTT NR8383 5 μg mL^{-1} *V* > 70%; 10 μg mL^{-1} *V* > 40% for all samples; WST NR8383 100 μg mL^{-1} *V* > 70% for all	A549; rat macrophages (NR8383)	2007	246
SWCNT	NR, MTT	24 h	5 μg mL^{-1} *V* > 75%	HUVEC	2006	247
SWCNT	NR, MTT	24 h	—	HUVEC	2006	248
SWCNT	NR, MTT	96 h	10 μg mL^{-1} *V* 100%	hMDM	2009	249
MWCNT	NR, live/dead	48 h	20 μg mL^{-1} *V* 80%	Macrophages	2011	250
SWCNT	NR; MTS	24, 48 h	EC_{50} = 316 μg mL^{-1} for 24 h; EC_{50} = 81 μg mL^{-1} for 48 h	HUVEC	2011	251
MWCNT	NR, MTT; live/dead	96 h	20 μg mL^{-1} MTT *V* 50%; other tests *V* 70%	hMDM	2009	252
SWCNT	NR, MTT, alamar blue; aqueous one (AQO)	24 h	0.4 μg mL^{-1} *V* > 60% (AQO)	HEK keratinocytes	2009	253
SWCNT	Optical microscopy	12, 24, 48, 60 h	50 μg mL^{-1} *V* 100%	HeLa	2007	254
MWCNT	PI	6 h	50 μg mL^{-1} *D* 30%	A549	2017	255
SWCNT; MWCNT	PI, acridine orange	12, 48 h	50 μg mL^{-1} *V* > 80%	H1299	2019	256
MWCNT	Resazurin assay	24, 48, 72 h	Raw IC_{50} > 80 μg cm^{-2}	Raw264.7; A549; Calu-3; alveolar macrophages	2016	257
SWCNT	Trypan blue	24, 48, 72 h	0.2 mg mL^{-1} *V* > 70% for 24 h and 72 h	Rat heart cell	2005	258
MWCNT	Trypan blue	24, 48, 72, 96, 120 h	10 ng per cell 24 h *V* > 80%, 48 h *V* 60%	T Lymphocytes	2006	259
MWCNT	Trypan blue	18 h	40 μg cm^{-2} *V* 70%	A549	2008	260
MWCNT + SWCNT	Trypan blue	24, 48, 72 h	24 h 100 μg cm^{-2} *V* 30%	BEAS2B	2009	261
MWCNT	Trypan blue	24 h	100 μg mL^{-1} *V* 60%	HUVEC	2011	262
MWCNT SWCNT	Trypan blue	24, 48, 72 h	24 h 100 μg mL^{-1} *V* 50%	Raw264.7	2011	140
SWCNT MWCNT	Trypan blue	24, 48 h	48 h BEAS2B 152 μg mL^{-1} *V* < 50% (SW); (SW) MeT5A 152 μg mL^{-1} *V* < 50%; (MW) MeT5A 152 μg mL^{-1} *V* 70%	BEAS2B; MeT5A	2013	263
SWCNT	Trypan blue	36, 120 h	30 μg mL^{-1} *V* near 80% for both times	A549	2013	264

Table 2 (*Contd.*)

Type	Cytotoxic test	Incub. time	Dose, viable (*V*)/dead (*D*) cell	Cell type	Year	Ref.
SWCNT	Trypan blue	24, 48 h	70 μg mL^{-1} *V* 20%	NIH3T3	2015	265
MWCNT	Trypan blue	13 w	1.92 μg cm^{-2} *V* near 100%	HBEC-3-KT	2018	266
MWCNT	Trypan blue	24, 48 h	60 μg mL^{-1} *V* > 60%	Primary microglial cells	2018	267
MWCNT	Trypan blue	24 h	0.1 mg mL^{-1} *V* > 70%	HUVECs; human liver hepatocellular carcinoma	2020	268
MWCNT	Trypan blue, EZ-cytox assay	24 h	EC_{20} for pristine 3 mg mL^{-1}; EC_{20} for OH near 6 mg mL^{-1}	BEAS2B; HepG2	2016	269
MWCNT (BP)	Trypan blue, NR	48, 72 h	—	Blood lymphocytes	2015	270
MWCNT + fibers	Trypan blue, MTT	24 h	1 mg mL^{-1} *V* 80%	NIH3T3	2008	271
MWCNT	Trypan blue, LDH	24 h	125 μg cm^{-2} *V* 20%	Mesothelial cells	2008	272
SWCNT	Trypan blue, LDH	24, 48, 72 h	50 μg mL^{-1} *V* 90%	T Lymphocytes	2008	273
MWCNT	Trypan blue, live/dead assay	24, 48, 72 h	72 h 50 μg mL^{-1} *V* 100%	THP-1	2016	274
MWCNT	Trypan blue, WST1	24, 48, 72 h	72 h 100 μg mL^{-1} *V* 50%	HPBCs (human peripheral blood cells)	2016	275
MWCNT	Trypan blue, WST1	24 h	A549 40 μg mL^{-1} *V* 80% (MTT 50%); BEAS2B 40 μg mL^{-1}; *V* 80% (MTT 50%)	A549; BEAS2B	2016	131
MWCNT	Trypan blue, WST8, CCK8	168 h	100 μg mL^{-1} *V* 100%	Dendritic cells	2009	276
MWCNT	WST1	24 h	GI_{50} = 0.0135%	NHBE	2012	277
SWCNT	WST1	72 h	20 μg mL^{-1} *V* 100%	hMSCs	2019	278
MWCNT (+fibers)	WST1	24 h	24 μg mL^{-1} *V* > 60%	BEAS2B	2020	279
MWCNT	WST1, CCK8	24 h	100 μg mL^{-1} *V* 70%	Human bone osteosarcoma; human gingival fibroblasts (HGF-1)	2016	280
MWCNT SWCNT	WST1, LDH	24 h	150 μg per 10^6 cells *V* 60%	HAEC	2009	281
MWCNT	WST8	24 h	IC_{50} = 12 μg mL^{-1}	BEAS2B; CHO-K1	2010	282
MWCNT	WST8	24 h	EC_{50} near 30 μg cm^{-2}	A549; HepG2	2019	283
MWCNT	WST8, MTT	16, 32 h	24 h LC_{50} = 26 μg mL^{-1}; 32 h LC_{50} = 22 μg mL^{-1}	J774.1; CHO-K1	2008	284

reaction between reagent and CNTs, which results in color changes and false viability assessment[131–133]

Fig. 4 displays the data of the toxicity of CNTs in dispersion as summarized in Table 2. Viability represents the percentage of alive cells after incubation time using a specified dose of CNTs. The dose is presented in μg mL^{-1} units using logarithmic scale (Fig. 4A and B), while incubation time indicates the duration of cells contact with the material. For both types of tubes, we have found only several studies where effects for SWCNTs and MWCNTs were similar in terms of cell viability.[134–144]

Earlier, it was shown that the composition of cell culture media and presence of bovine serum protein might strongly affect tube agglomeration, their bioavailability in dispersion and internalization in cells.[82,138,145] We included different cell types in our analysis, so culture media might vary for cells cultures.

After dividing cells according to types of tissue, we realized that studies of connective tissue demonstrated high values of the CNT toxic impact (Fig. 4A). Researchers also registered small viability for epithelial tissues in some unique cases, but this is much rarer than in case of the connective ones.

To present the found data in a simplier way, we normalized the dose added to cells by incubation time. By using Spearman statistical criteria for analysis of relations between viability and doses, we have found a slight correlation between cells viability and dose per incubation time ($r = -0.1648$), *i.e.*, a dose of tubes per hour (μg mL^{-1} h^{-1}), (number of studies (N) = 214; statistical significance (p-value) = 0.0158). Cells viability and dose/incubation time parameters are weakly negatively correlated, meaning that dose increase results in the decrease of viability. We propose that the different dilution procedures could explain the absence of strong correlations. Undoubtedly, this is a rough estimation because cells meet the entire dose at the first interaction moment at incubation. However, cell adaptation to a prolonged regime may affect cells survival, so we also used dose/time characteristics for the analysis.

We further extracted only IC_{50} (inhibitory concentration) values, the mean value for studies with the known IC_{50} dose was 3.9 μg mL^{-1} h^{-1} (N = 60, 27.9% out of the entire sample); by calculating for 24 hours, it would be 93.8 μg mL^{-1}.

By analyzing the extracted data in terms of frequency distribution for dose/time and viability parameters (Fig. 4C–E)

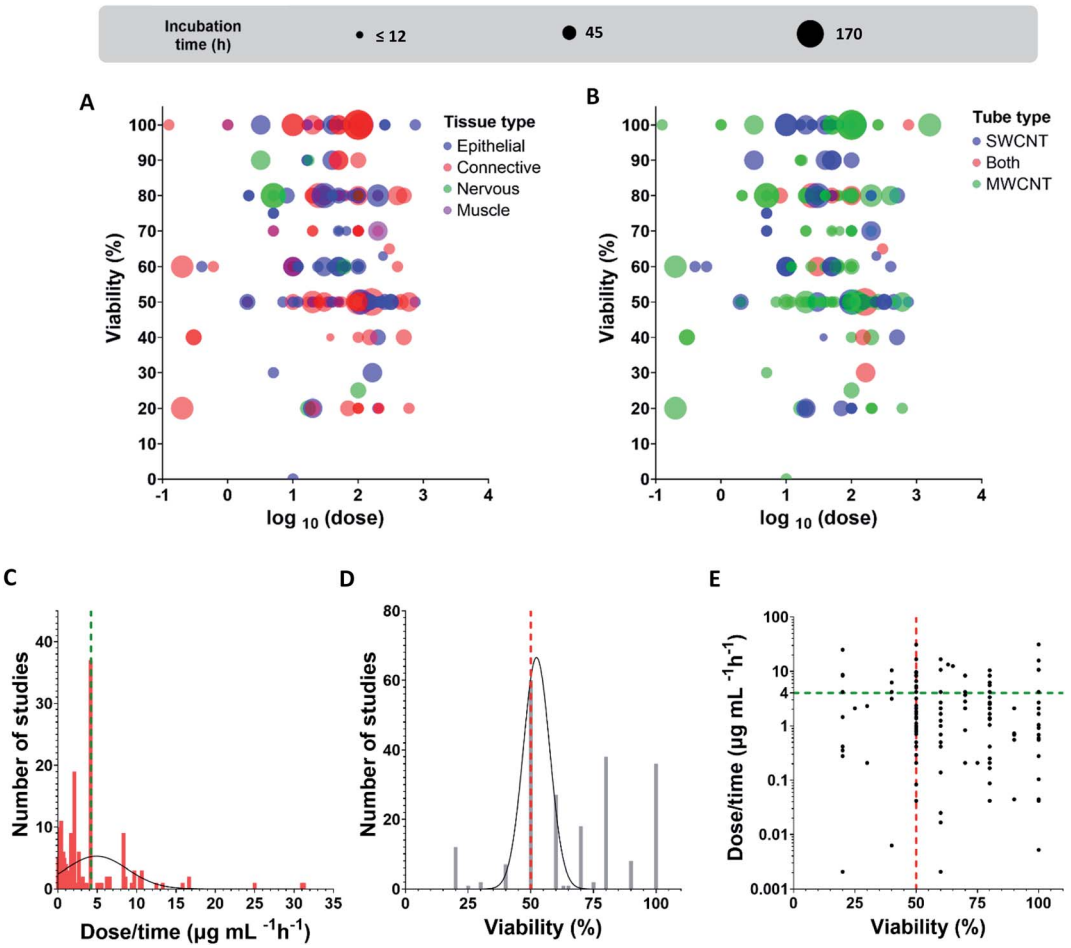

Fig. 4 Dose (log(μg mL^{-1})) dependence of viability (a percentage of alive cells after incubation) at various incubation time for (A) different tissues (blue – epithelial, red – connective, green – nervous, purple – muscle), (B) CNTs type (blue – SWCNT, green – MWCNT, red – both). Frequency distribution for (C) dose/time values (μg mL^{-1} h^{-1}) (black line is a Gaussian distribution, green line shows the maximum), (D) viability values (black line is a Gaussian distribution, red line shows the peak rounded up value). (E) Viability *versus* dose/time (green and red lines position the maximum frequencies crossing for both characteristics (C and D), viability and dose/time).

we found that the majority of studies use IC_{50} values for cytotoxic analysis (27.9% out of the all revealed studies). Moreover, the most studied dose/time values are exactly in the region 4–5 μg mL^{-1} h^{-1} which is close to the found mean value for the viability 50% (3.9 μg mL^{-1} h^{-1}). By applying a Gaussian distribution, we also revealed the maximum frequencies for the distribution given in Fig. 4C and D; for viability parameter, the maximum of distribution is 52% (amplitude 66.6, mean 52.3, SD 5.3), for dose/time, the maximum is precisely in the range 4–5 μg mL^{-1} h^{-1}(amplitude 5.3, mean 4.9, SD 3.9). The intersection of green and red lines in Fig. 4E positions the maximum frequencies crossing for both characteristics, viability and dose/time; the maximum dots density is localized precisely in that region.

To sum up, we suggest CNT IC_{50} toxicity dose to be about 4–5 μg mL^{-1} h^{-1}. Based on the found data, we also propose that the CNT toxic range is comparable with doxorubicin, a well-known agent for chemotherapy treatment (IC_{50} < 10 μg mL^{-1} for some cell lines after 24 h incubation).[95,146–148]

Conclusions

Thus, CNTs on a substrate are safe for many cell types. Nearly 90% of the publications included in the analysis reported the absence of a negative impact on cell cultivation on the CNT substrates.

In the case of dispersions, we propose toxicity values comparable with the toxicity of a well-known chemotherapy agent called doxorubicin (IC_{50} < 10 μg mL^{-1}). During our studies, we also identified the most suitable cell models (A549 and HUVECs) for testing the CNT toxicity and the comparison among research groups. Despite the examined phenomena of false-negative and unreliable results for cell viability obtained by the MTT test, near 26% of all works used the colorimetry assay to test cell viability.

There is no doubt that such CNT parameters as diameter, length, purification procedure, and synthesis may greatly affect toxicity, and should be carefully studied further employing a similar systematic approach.

The authors see an urgent need for standardization of materials and methods to investigate them. Guidelines such as OECD principles for proper laboratory practice are highly expensive and time consuming, therefore there is a strong need for a more convenient universal approach for testing the materials safety or toxic impact in a regular laboratory practice. Demonstrated results imply a need for practical toxicity assessment of CNTs with different geometry and functionalization, to deepen the understanding of what affects the CNT toxicity.

List of abbreviations

4-HNE	4-Hydroxynonenal
Akt	Type of serine/threonine protein kinase
AP-1	Activator protein 1
CCK	Cell counting kit
CNT	Carbon nanotube
CVD	Chemical vapor deposition
EN	Ethylenediamine
FDA	Food and Drug Administration
HiPCO	High pressure carbon monoxide
ISO	International organization for standardization
LDH	Lactate dehydrogenase
MAPK	Mitogen-activated protein kinase
MTS	3-(4,5-Dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl-2*H*-tetrazolium bromide
MWCNT	Multiwalled carbon nanotube
NF-kβ	Nuclear factor kappa-light-chain-enhancer of activated B cells
NLRP3	Nod-like receptor family pyrin domain containing 3
NR	Neutral red
OECD	Organization for economic co-operation and development
p53	Tumor protein
PEG	Poly(ethylene oxide)
PEI	Poly(ethyleneimine)
PI	Propidium iodide
SWCNT	Single-walled carbon nanotube
TGF-β	Transforming growth factor beta
WST	4-[3-(4-Iodophenyl)-2-(4-nitro-phenyl)-2*H*-5-tetrazolio]-1,3-benzene sulfonate
XTT	Sodium 3′-[1-(phenylaminocarbonyl)-3,4-tetrazolium]-bis (4-methoxy6-nitro) benzene sulfonic acid hydrate

Author contributions

Margarita R. Chetyrkina carried out the literature search, wrote the initial manuscript and made subsequent modifications. Fedor S. Fedorov carried out the literature search, participated in writing and editing the manuscript. Albert G. Nasibulin supervised the work, participated in writing and editing the manuscript.

Conflicts of interest

There are no conflicts to declare.

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