

Chapter 2

Carbon Nanotubes: Biorisks and Biodefence

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Abstract From the time of the discovery of carbon nanotubes (CNT) the question of their toxicity remains of key importance. The practical use of these unique nanomaterials in biotechnology, molecular biology and medicine can be complicated because of possible adverse effect of CNT on subcellular and cellular structures, tissues and whole organs. Similarly to any other nanoparticles, CNT toxicity is defined by their form, size, purity, charge, dose, entry route into the body, concentration in the target organ, duration of contact and other factors. The research on toxicity and biocompatibility of CNT of different origin, structure and purity began in 2001. It has been conducted with various biological components, performing experiments both *in vitro* and *in vivo*.

Here some aspects of cytotoxicity and potential for medical use of CNT are discussed.

Keywords Carbon nanotubes • Cytotoxicity • Cytocompatibility • Medical applications of CNT

2.1 Introduction

From the time of Iijima's publication on carbon nanotubes, CNT [1] (which were probably discovered earlier [2]), the question of their toxicity remains of key importance. The practical use of these unique nanomaterials in biotechnology, molecular biology and medicine can naturally be complicated because of possible

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adverse effect of CNT on subcellular and cellular structures, tissues and whole organs. Similarly to any other nanoparticles, CNT toxicity is dependent on their shape, size, purity, charge, dose, entry route into the body, concentration in the field of body-target, duration of influence and other factors. The research on toxicity and biocompatibility of CNT of different origin, structure and chemical purity has been performed since 2001. It has been conducted with various biological components, performing experiments both *in vitro* and *in vivo*. A number of original papers and reviews on this subject are available [3–8].

Carbon nanotubes are among the most interesting objects of nanotechnology. They have cylindrical structure with a diameter in the range of one to several dozen nanometres and length of several nanometres to several microns. CNT are built of one or several graphene layers with hexagonal arrangement of carbon atoms. Tubes have a tip in the shape of a hemispherical head with a chemical structure of a half fullerene. Unlike fullerenes, which represent the molecular form of carbon, CNT combine properties of nanoclusters and a massive solid body. This leads to an occurrence of specific, sometimes unexpected mechanical, optical, electric, magnetic and physico-chemical properties, which attract the attention of researchers and end-users:

Mechanical properties: hardening of metals and alloys, creation of novel polymeric composites, special additives to lubricants and oils, etc.

Electronic properties: semiconductor and metal conductivity, magneto-resistance, emission of electrons, electronic devices of the molecular size, information recording, diodes, field transistors, cold cathodes, materials for displays, quantum wires and dots, cathodes for X-ray radiation, electric probes, etc.

Optical properties: light-emitting diodes, resonance absorption of near IR-radiation;

Physical and chemical properties: large specific surface and possibility of surface chemical modification, adsorbents, catalysts, chemical sensors, materials for electrodes, chemical batteries, fuel elements and super condensers.

Biological properties: ability to migrate into biological cells, biosensors, prosthetics, drug delivery, medical nanodevices, application in gene engineering.

The industrial production of CNT has now achieved several tons per year and it is continually increasing. During their production, conditioning and applications, CNT can penetrate into the human body by inhalation, contact with skin, with food and drinking water, or deliberate introduction into the blood and under the skin if used for medical applications. They can also influence microorganisms, plants, animals, when they are released into the environment in significant amounts. However despite the vast knowledge generated about CNT, their impact on biological objects is still not clear.

2.2 Biorisks Associated with CNT

A variety of new materials had been produced using nanoparticles before scientists became concerned about their possible negative impact and consequences for the human body and the environment. The toxicology of carbon nanomaterials,

in particular nanotubes, has emerged only recently and it is at a stage of accumulating primary data. There are three main factors which define the ability of carbon nanoparticles to cause possible damage:

- A high surface area-to-mass ratio and as a result a large area of contact between the nanoparticle and the cellular membrane, and significant influence on adsorption and transport of toxic substances.
- Contact time: the longer CNT contact with the cell membrane, the higher probability of cell damage. However, this factor also includes the mobility of nanoparticles, which can migrate to the surrounding tissue and be removed from the organism.
- The reactivity or inherent toxicity of chemical substances introduced together with CNT. They can be located inside nanotubes or attached to their external surface. This factor is related to the surface area-to-mass ratio of nanoparticles. The higher the ratio, the more likely is the negative impact.

Studying toxicity and biocompatibility of CNT is very important. Smart et al. [9] outlined directions of future research in this field. It includes pulmonary toxicity, skin irritability, macrophage response, interrelation of CNT with their toxicity, absorption, distribution and excretion, and influence of chemical functionalization of CNT on their biocompatibility.

It is not clear yet to what extent the mechanical damage of cell membranes caused by nanotubes and the effect of CNT on biochemical processes in subcellular organelles (mitochondria and nuclei) contribute to nanotubes toxicity. While the influence of CNT on DNA and cell nuclei was experimentally proved [10, 11], there is no data on the effect of nanotubes on the activity of mitochondria, which play a key role in viability of cells. The data currently available on pulmonary toxicity, skin irritability, cytotoxicity, biocompatibility, influence on environment, and therapeutic action of CNT are inconsistent and do not give a clear picture about level of safety of such nanomaterials for living organisms. It has become clear only that purified single-walled nanotubes of small length and chemically modified nanotubes with functionalized surfaces have lower toxicity and better biocompatibility. It is known that CNT are capable of entering the membrane of biological cells, to reach the cytoplasm, and in some cases into the nucleus. It has been established empirically that concentration limit of cytotoxicity of CNT suspension is about 0.01 mg/mL.

Some data on CNT cytotoxicity and cytocompatibility are summarized in Tables 2.1 and 2.2 [12–28].

To study the mechanism of cytotoxic action of CNT we used a modified method of spin labels [20], which allows the quantitative determination of CNT influence on membrane integrity of human blood erythrocytes, and mitochondrial activity of hepatocytes in rat liver homogenate, without extraction of mitochondria from cells.

Water-soluble iminoxyl free radical – 2,2,6,6-tetramethyl-4-oxo-piperidin-1-oxyl (commercial trade name TEMPON) was used as a paramagnetic probe.

Table 2.1 Cytotoxicity of CNT

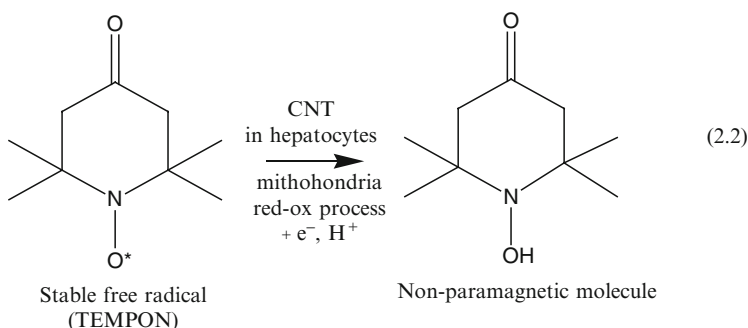
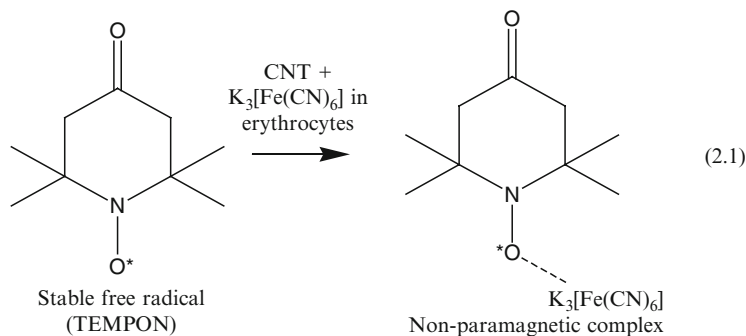
| Authors | Material | Type of cell | Result |
|---------------------------------|--|--|--|
| Shvedova et al. [12] | SW CNT, Fe-catalyst | Human keratinocytes (HaCaT) in solution with 0.06–0.24 mg/mL CNT, contact time 8 h | Accelerated oxidative stress (production of free radicals and peroxides, exhaustion of general antioxidant reserves); decrease of cell viability, morphological changes |
| Monteiro-Riviere et al. [13] | MW CNT, (CVD- method), purified | Human keratinocytes of (HEK) in solution with 0.1– 0.4 mg/mL CNT, contact time 48 h | Production of inflammatory cytokines (IL); reduction of cells viability depending on time and dose of exposure |
| Tamura et al. [14] | CNT, purified | Human blood neutrophils in contact with CNT for 1 h | Increase of superoxide anion-radicals and inflammatory cytokine (TNF- α) production; decrease of cells viability |
| Cherukuri et al. [15] | SW CNT, purified | Phagocytic cells of mice (J774.1) | Catching ~50% of nanotubes, no cytotoxic effect |
| Shvedova et al. [16] | SW CNT, Fe-catalyst | Macrophagic murine cells (RAW264.7) | Increase of pro-fibrotic mediator TGF- β 1; no oxidative burst, nitric oxide production or apoptosis was observed |
| Muller et al. [17] | MW CNT, purified | Peritoneal macrophages of rats – incubation in solution with 20, 50 and 100 mg/mL CNT, contact time 24 h | Release of lactate dehydrogenase and inflammatory cytokines (mRNA squirrel TNF- α) |
| Jia et al. [18] | SW and MW CNT (arc, CVD), purified | Alveolar macrophages – solution of SW CNT (conc. 1.41– 226 mg/cm ²) and MW CNT (conc. 1.41–22.6 mg/ cm ²), contact 6 h | Reduction of cells viability and macrophages functional ability decrease |
| Cui et al. [19] | SW CNT | Human embryonic kidney cells (HEK 293) in solution with 0.78–200 mg/ mL of SW CNT | Induction of apoptosis and reduction of adhesion ability (and corresponding genes), reduction of cellular proliferation |

SW- single wall, MW- multiwall

Table 2.2 Cytocompatibility of CNT

| Author | Material | Type of cell | Results |
|---------------------------|--|---|--|
| Elias et al. [21] | CNT-containing orthopedic materials | Osteoblasts – inoculation on material | Increase of osteoblast proliferation, increase of alkaline phosphatase activity, absence of cytotoxicity |
| Supronowicz et al. [22] | PLA/CNT – nano-composites | Osteoblasts – contact with nanocomposites, effect of electric current | Increase of osteoblast proliferation |
| Price et al. [23] | PU/CNT – nano-composites | Osteoblasts, chondrocytes, fibroblasts, plain muscular cells – contact with PU/CNT | Adhesion increased only for osteoblasts; absence of cytotoxicity |
| Correa-Duarte et al. [24] | MW CNT; oxidized | Fibroblasts of mice (L929) – inoculation to CNT, observation – 7 days | Formation of the isolated cells, fusion after 7 days, absence of cytotoxicity |
| McKenzie et al. [25] | MW CNT; purified, different diameter | Astrocytes (cells which are responsible for reduction of nervous tissue damages) – contact with CNT surface | Normal proliferation, adhesion and functional activity on tubes, especially with a diameter less than 100 nm |
| Hu et al. [26] | PU/CNT – nano-composites | Astrocytes, axons of rats – contact with PU/CNT nanocomposites | Reduction of astrocyte adhesion, increased inhibition of axons. Cytotoxicity is absent |
| Gabay et al. [27] | MW CNT; purified and chemically modified | Neurons – inoculation on CNT; observation – 4 days | Localization on CNT, proliferation of axons. Cytotoxicity is absent |
| McKnight et al. [28] | Vertically oriented nanofibers + DNA | Chinese hamster ovary (CHO) cells – centrifugation and compression | Part of cells was lost, small production of GFP; cytotoxic response was not observed |

PLA - polylactic acid, PU - polyurethane



ESR-spectra of TEMPON degradation in erythrocytes and hepatocytes were studied. The degradation was caused by the chemical processes presented in Schemes 2.1 and 2.2.

The erythrocyte membrane damage caused by CNT increased with time (Fig. 2.1a). Introduction of CNT suspension at concentrations from 0.01 to 0.2 mg/mL during the first step did not lead to erythrocyte membrane infringement. However after 2 days of exposure to CNT at concentrations ranging from 0.01, 0.05, 0.1 and 0.2 mg/mL and at temperature 6°C the quantity of damaged erythrocytes was 4, 10, 16 and 25%, respectively.

The incubation of liver homogenate with CNT for 4 h at 0°C leads to considerable decrease of mitochondrial activity (perhaps due to inhibition of chain transfer of electrons in mitochondria). The data obtained showed (Fig. 2.1b) that the cytotoxicity caused by CNT is associated with not only structural changes in the cell membrane, but also with CNT influence on their functional properties.

We have studied CNT influence on growth rate and proliferation of some cellular colonies [29]. Interesting results were obtained in case of bread-making yeast-like fungi *Saccharomyces cerevisiae* (strain 608) and hamster kidney cells. Introduction of small amounts of CNT (~3 µg/mL) in fungal suspensions led to 2-fold increase in *Saccharomyces cerevisiae* colonies number compared to the control, after 48 h of incubation at 30°C (Fig. 2.2). Similar results were obtained for colonies of hamster kidney cells. Presence of CNT activated cell proliferation and increased cell growth rate by 1.5 times.

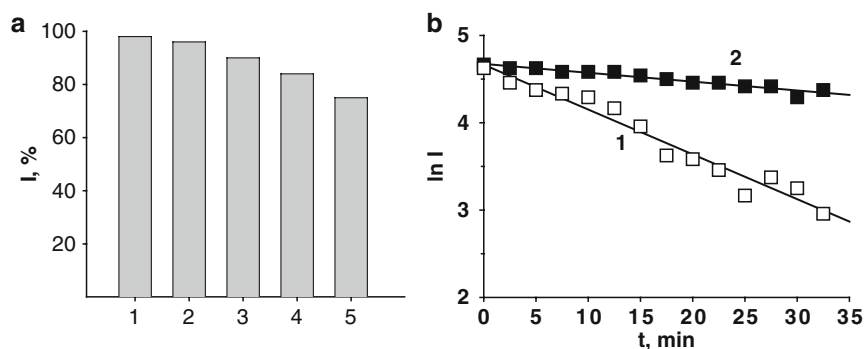


Fig. 2.1 (a) Influence of CNT concentration in presence of $K_3[Fe(CN)_6]$ on intensity of the ESR spectra of the paramagnetic label TEMPON: control (without CNT) (1) and adding CNT to blood erythrocytes at 0.01 (2), 0.05 (3), 0.1 (4) and 0.2 (5) mg/mL; (b) kinetics of the ESR signal intensity decay of the paramagnetic label in liver homogenate after 4 h inoculation: 1 – control (without CNT), 2 – in presence of CNT with concentration 0.2 mg/mL

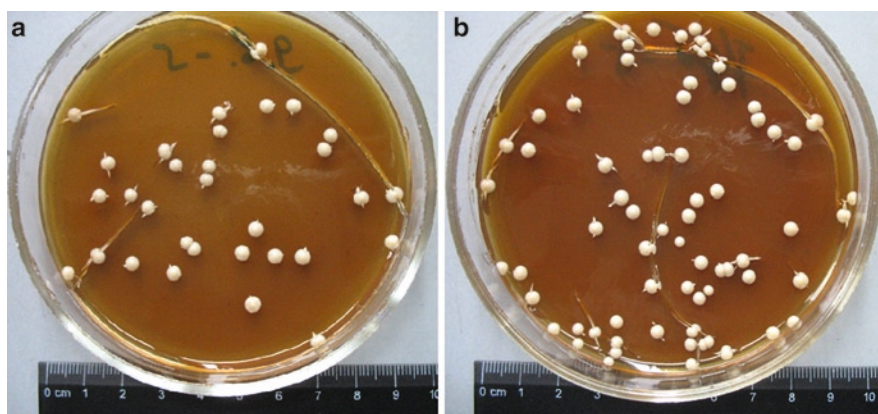


Fig. 2.2 Influence of adding CNT into a nutrient medium on growth of *Saccharomyces cerevisiae* colonies after 48 h of incubation: (a) control (without CNT), (b) in the presence of CNT ($\sim 3 \mu\text{g/mL}$)

2.3 Biomedical Applications of CNT

Nanotubes are functionalised to improve their solubility in water or to attach to their surface biologically active substances such as peptides and drugs. The ability to attach biological substances has raised an interest in using nanotubes as carriers for delivery of drugs and vaccines. A number of researchers performed functionalization of CNT with physiologically active molecules and macro-objects. These results are summarized in Table 2.3 [30–40].

Compared to classical drug delivery systems such as liposomes or peptides, nanotubes have a higher efficiency [41] and this can be used for the further development of delivery systems. Stability and diversity of nanotube forms provide long time circulation and biocompatibility that result in more efficient transport of substances.

Table 2.3 Use of CNT as carriers of bioactive substances

| Author | CNT conjugate | Results |
|------------------------|---|---|
| Pantarotto et al. [30] | Functionalized SW CNT+small peptide sequence from the foot-and-mouth disease virus (FMDV) | SW CNT-FMDV peptide complex induced a specific antibody response <i>in vivo</i> . It was maintained and recognized by mono- and polyclonal antibodies |
| Pantarotto et al. [31] | Functionalized SW CNT+peptide fragment from the α -subunit of the Gs protein (α s) | SWCN- α s complex was able to cross the cellular and nuclear membranes (human 3 T6 and murine 3 T3 cells) |
| Kam et al. [32] | Purified and shortened SW CNT+streptavidin | SW CNT-streptavidin conjugate caused extensive cell death, which was attributed to the delivery of streptavidin to the cells (proleukemia cells of human and T-lymphocytes) |
| Wu et al. [33] | CNT+amphotericin B | Amphotericin B entered various cells and increased its activity |
| Bianco et al. [34] | CNT+proteins (fibrinogen, protein A, erythropoietin, and apolipoprotein) | CNT-TEG-short protein complex quickly entered fibroblasts and other cells, sometimes migrated to their nuclei. Proteins executed their normal biological functions |
| Lu et al. [35] | SW CNT+RNA polymer | Successful transportation of SW CNT-RNA polymer complex into cytoplasm and nucleus of cell |
| Pantarotto et al. [26] | SW CNT and MW CNT+plasmid DNA | All conjugates influenced regulative expression of marker genes in human cells |
| Cai et al. [37] | SW CNT+plasmid DNA, with nickel under the influence of a magnetic field | High efficiency of transduction of SW CNT-DNA conjugates in lymphoma cells (Ball 7 B-lymphoma) |
| Kam et al. [38–40] | SW CNT+cytochrome C, RNA, DNA | CNT transferred cytochrome C to the cancer cells; accumulation of SW CNT-RNA conjugates in cytoplasm and nucleus of HeLa cells |

By using nanotubes for drug delivery the problem of poor solubility of a considerable number of substances such as many medicines could be overcome. Furthermore, nanotubes can be modified to improve their contact and penetration into target cells. In conclusion, using nanotubes for drug delivery should increase efficiency of the latter and can reduce their side-effects.

SW CNT functionalized with DNA showed a 10 times more effective penetration and expression of genes *in vitro*, in comparison with molecular DNA. Other charged macromolecules such as polypeptides and liposomes, can provide more effective transport, but they can cause destabilization of the cellular membrane exhibiting a cytotoxic effect. Whereas, using nanotubes for gene delivery has not caused any cytotoxic effects.

Successful gene therapy demands an effective system for therapeutic gene delivery into organs and tissues. Therefore gene delivery is based on the development of a non-viral delivery system. Such vector systems have the ability to introduce the alien genetic information into a cell. Carbon nanotubes can be used for creation of new vectors for gene transportation.

2.4 CNT: Pros and Cons

Carbon nanotubes are unique materials with specific properties [42]. There is a considerable application potential for using nanotubes in the biomedical field. However, when such materials are considered for application in biomedical implants, transport of medicines and vaccines or as biosensors, their biocompatibility needs to be established. Other carbon materials show remarkable long-term biocompatibility and biological action for use as medical devices. Preliminary data on biocompatibility of nanotubes and other novel nanostructured materials demonstrate that we have to pay attention to their possible adverse effects when their biomedical applications are considered.

Despite the need to know how nanotubes may affect or cause toxicity for live organisms, only a small number of studies have been dedicated to this problem. Furthermore, results of these studies have been inconsistent and not fully understood. The data obtained show that crude nanotubes possess a certain level of toxicity (in both *in vivo* and *in vitro* studies) associated mainly with the presence of metals, which are used as catalysts in nanotube synthesis. For purified nanotubes minimal toxic effects were seen even at high concentrations, and chemically functionalized nanotubes used for drug delivery did not show any toxic effects. However, the ability of nanotubes to form aggregates requires further research in this area.

From the data obtained so far one could conclude that work with nanotubes should be done with precaution, and certain safety actions need to be considered working with nanotubes in laboratories and during their manufacture. The success of nanotechnologies will depend on continuing research in the area of toxicology of carbon nanotubes and the materials based on them.

In the study conducted by Hurt et al. [43], toxicology of nanoparticles (nanotoxicology) with a special emphasis on CNT was considered. The necessity of carrying out toxicology research as well as the lack of such work in this area was highlighted. It was considered that the future development of nanotoxicology is associated with the following:

- Materials should be characterized and described in as many details as possible, because the nanotube toxicity can depend on by-products of their synthesis as well as on their design. It would be desirable to provide at least information on their composition (including metals and heteroatoms, which are present in a quantity higher than 0.1%), detailed description of morphology, data on surface chemistry, crystallinity, and spatial organization of graphene planes;
- Better understanding of mechanism of nanotube interaction with biological objects is required; the possible toxicity will depend on dose and exposure time;
- Methods for tracking nanotubes in biological materials are needed to measure the rate of nanotube transport, distance of penetration from places of introduction at inhalation, with food and water, and implantation of nanotubes;
- Methods for dose measurement;
- Determination of the main indicators of toxicity, as carbon nanotubes can cause different toxic effects.

It was concluded that the overarching objective of nanotube toxicology is to find materials that will have no harmful effect on nature and humans.

The progressive growth of technologies of production and applications of nanomaterials, in particular on the basis of nanocarbons (fullerenes, nanotubes, nanodiamonds, aerogels, etc.) is observed all over the world. Physical, chemical and mechanical properties of such substances are capable of exerting an unpredictable impact on biological objects. In this review, we have offered approaches to formation of identification methodology, toxicological research and assessment of risks for human organisms and the environment posed by manufacture and use of nanosized substances [44]. In course of our study, the experience gained from the Chernobyl catastrophe, and the effect of small doses and low intensity of technogenic pollution on a human body has been used.

It is obvious that we are at the stage of accumulating the knowledge of how to handle safely nanosized objects. Carbon nanotubes are currently and will be in the future at the forefront among other known nanomaterials, in terms of volumes of research, manufacturing and applications in various fields of practical activities, including medicine and biology.

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