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Carbon nanotubes: properties, applications and toxicity

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Abstract Carbon nanotubes (CNTs) are represented by sheets of graphite with tubular shape, that possess excellent mechanical strength and elasticity. They are highly investigated worldwide and outstanding properties and applications in biological and medical domains were reported since their discovery in 1991. The most important details regarding the history, types, structure, synthesis and properties of CNTs are highlighted in this review. The functionalization represents a process required to increase the solubility and biocompatibility of CNTs by adding hydrophilic groups on their surface. Drug delivery represents one of the most important applications in medicine of CNTs, being used as transporters for various biological molecules. Despite the tremendous achievements obtained for the biomedical applications, the cytotoxicity of CNTs remains a debate issue, being influenced by various factors, such as presence of impurities, functionalization, diameter, concentration, exposure time and cell type.

Keywords: carbon nanotubes, properties, functionalization, drug delivery, cytotoxicity

General aspects of carbon nanotubes

1. Brief history

Carbon nanotubes (CNTs) are tubular-shaped carbon molecules with special properties which enable their usage for a plenty of biomedical applications. Although nowadays CNTs are highly investigated by many researches, CNTs had not been fully appreciated at the beginning of their story. The first observation of a new carbon fibre was made in the late 1950s by Roger Bacon at Union Carbide (Bacon, 1960), but just after almost 20 years Morinobu Endo noticed tubes consisting in a single layer of rolled-up graphite (Oberlin et al., 1976). In the context of fullerenes discovery in 1985 (Kroto et al., 1985) other independent CNT studies emerged, as Sumio Iijima from NEC Corporation and Donald Bethune at IBM were able to report independently in 1993 the observation of single-wall nanotubes, named "buckytubes", (Bethune et al., 1993; Iijima and Ichihashi, 1993). The year 2004 marked the outstanding achievement of technology development which led to the production of an individual 4 cm long single-wall carbon nanotube (SWCNT), its photo being published by the prestigious Nature journal (Zheng et al., 2004). The most important discoveries and outstanding researches on CNTs were summarized in Table.

Since 2005, CNTs started to be used in various fields, such as electronics, materials science, chemical processing, energy management and healthcare. The most outstanding innovations and scientific breakthroughs based on CNTs across the years include:

(1) the development of high-definition flat screen (first time reported in 2005 – "New Scientist" magazine) using carbon nanotube field emission display (CNT FED TV) which provides great advantages such as low consumption of power, higher brightness, a wider viewing angle, faster rate of response and a lower cost of manufacturing;

(2) the fabrication of an ideal CNT diode with improved photovoltaic effect to get more effective solar cells (first time obtained by General Electric in 2005 and further improved by Strauf and collaborators in 2013);

(3) the regeneration of the optic nerve using CNTs as scaffolds (Ellis-Behnke et al., 2006);

(4) the synthesis of CNT transistors that surpass in performance state-of-the-art silicon transistors (in terms of five times faster or five times less energy compared to silicon transistors) (Brady et al., 2016).

2. Structure and classification

CNTs present a structure similar to fullerene, although the fullerene has a spherical form whilst nanotubes have cylindrical conformations having the ends coated by half of a fullerene molecule. Generally, the diameter of nanotube is in the nanometer range while the length can be up to a few millimeters. If we take into consideration that graphene is an individual graphite layer, it can be considered that CNTs are represented by sheets of graphite (hexagonal networks of carbon atoms) rolled into a tube, according to Thostenson et al., 2001. Similar to graphite, the characteristic chemical bonding between carbon atoms is sp^2, each atom joining to three neighboring carbon atoms and forming a tight network which provides the unique strength of CNTs. The carbon atoms align into ropes which are stabilized by van der Waals forces, resulting in very strong wires of nanometric size.

Table Timeline of the most important discoveries and ever-growing body of research conducted on CNTs

Year	Type of Discovery	References
1985	Discovery of Fullerenes	Kroto et al., 1985
1991	Discovery of MWCNTs	Iijima, 1991
1992	Conductivity of CNTs	Mintmire et al., 1992
1993	Structural rigidity of CNTs	Overney et al., 1993
1993	Synthesis of SWCNTs	Iijima and Ichihashi, 1993
1995	Nanotubes as field emitters	Rinzler et al., 1995
1996	Ropes of SWCNTs	Thess et al., 1996
1997	Quantum conductance of CNTs	Tans et al., 1997
1998	Synthesis of aligned CNT films	Ren et al., 1998
2000	Thermal conductivity of CNTs	Berber et al., 2000
2000	Macroscopically aligned CNTs	Vigolo et al., 2000
2001	Intrinsic superconductivity of CNTs	Kociak et al., 2001
2004	Nanotube molecular transporter	Shi Kam et al., 2004
2006	Nanotube-based sensor	Heller et al., 2006

There are different types of CNTs with various structures, depending on length, thickness, number of layers and how the graphene sheet was rolled up in order to form the tube (inducing a metallic or semiconductor property and generating three possible types of nanotubes – armchair, zigzag and chiral nanotubes). Generally, CNTs can be classified in two main categories: single-walled (SWCNTs) – with one wrapped graphene sheet in the form of a cylinder, and multi-walled (MWCNTs) – a collection of concentric SWCNTs of continuously increasing diameters held at a certain distance from each other by interatomic forces (Aqel et al., 2012). Also, there can be other rare types such as: fullerite, torus and nanoknot.

SWCNTs have diameters between 0.4 and 3 nm, while MWCNTs can vary from 2 to 100 nm. The length is generally of several micrometers, generating a length to diameter ratio of approximative 1000 for SWCNTs. The characteristic interlayer distance for MWCNTs is 3.3 Å which is almost similar to the one between graphene layers in graphite (Hou et al., 2003).

3. Synthesis and functionalization

There are three conventional methods of CNT synthesis: arc discharge (the first procedure which opened the research of CNTs all over the world), laser ablation and chemical vapor deposition (CVD) (Koziol et al., 2010). The last one is the most used method to obtain CNTs and involves a metal catalyst (e.g., Ni, Fe or Co) which reacts with a hydrocarbon source at temperatures between 500 and 1200°C, depending on the type of CNTs (low temperature of 600 – 900°C favors MWCNTs, while higher temperature yields SWCNTs growth). Although it is easily to produce at large scale MWCNTs, advances are still making to obtain kilograms of SWCNTs with control over their diameter (Sajid et al., 2016).

Although CNTs possess high surface area and mechanical strength, ultra-light weight, chemical and thermal stability and electronic properties, these nanomaterials show a lack of solubility in aqueous media which is a major obstacle limiting the array of applications of nanotubes (Yang et al., 2007). This important technical barrier was passed by adjusting the length size of the tubes to a maximum of 100-300 nm, but mainly by developing new methods able to chemically change and functionalize CNTs for a more facile processing in physiological environments (Sánchez-Pomales et al., 2009). Functionalization (surface attachment of specific organic groups) provides CNTs with novel properties, depending on the nature of the modification. For example, coating CNTs with hydrophilic macromolecules such as collagen (a type of non-covalent functionalization) increases resistance and

is used in three dimensional arrays (MacDonald et al., 2005). On the other hand, covalent functionalization can provide surface charge and reactivity changes which greatly expands the utility of this nanomaterial (Dyke and Tour, 2004), including in terms of exploiting it in biomedical applications. SWCNTs are better suited materials in this regard, having a higher surface area for interaction with enzymes, although MWCNTs are often chosen for their easier dispersibility and the low cost of production (Feng and Ji, 2011).

Depending on the type of biomolecule used for CNT bounding, three main approaches for the biomodification of CNTs were described in a previous review by Yang et al. (2007): (i) covalent attachment – represented by a chemical bound formation, the most common method being the reaction with carboxylic acid (–COOH) residues introduced by oxidation using strong acids; (ii) non-covalent attachment – represented by an adsorption of various molecules, such as aromatic and/or hydrophobic molecules (e.g., polyethylenglycol, pyrenes); and (iii) a hybrid approach – represented by a two-step process in which a small molecule is non-covalent anchored to CNTs and then covalently attached to the biomolecule of interest, such as: proteins, polysaccharides and DNA. The last two approaches have a major advantage compared to covalent attachment, represented by the fact that CNT structure is not significantly altered, although there is still a lack of specificity and sometimes the target biomolecule can be denaturated upon adsorption (Yang et al., 2007).

Functionalization provides novel properties to CNTs, depending on the nature of the modification. The high surface area of the nanomaterial allows multiple attachment sites for cell targeting agents, thus making CNTs potential ideal candidates for targeted drug delivery systems. Such functionalized CNTs are more compatible with aqueous environments, priming them for interactions within biological systems and future exploitation in biomedical applications. For example, in order to diminish immunogenicity or nonspecific cellular uptake, CNTs designed for drug delivery are generally functionalized with polyethylene glycol (PEG) weighting between 1 and 40 kDa (Lamprecht et al., 2012).

4. Physicochemical properties

The major physico-chemical properties of CNTs were previously discussed by Kaushik and Majumder, 2015). Briefly, the CNT has excellent mechanical strength and elasticity, due to its hexagonal structure and the sp2 chemical bonds between the carbon atoms (π electrons conjugation). The density of this nanomaterial can be as low as 1.3 g/cm^3, which is one-sixth that of stainless steel, and a tensile strength of up to 63 GPa makes CNTs 50 times stronger than steel and the strongest material ever discovered. Its stiffness (measured by Young's moduli, > 1 TPa) also makes it superior to steel or any other carbon fibre (Saifuddin et al., 2013).

Also, they have unique electrical properties – compared to other conductive polymers, and retain their electrical conductivity under harsh conditions, which make them suitable for inducing an electrical charge and polarization of cells. This property is particularly important to electro-active cells where electrical stimulation (ES) is employed to engineer corresponding tissues and regenerate action potentials in vitro (Ramón-Azcón et al., 2014). Low frequency ES is of great importance for various cellular behaviours (i.e., alignment, differentiation, metabolic activity, protein synthesis).

High optical absorbance in the near-infrared region is another important property of CNTs, making them detectable by infrared fluorescence microscopy and cell labeling (Jain et al., 2015). In addition, high chemical stability and thermal conductivity, and low thermal expansion coefficient are characteristics of CNTs, valuable for biomedical purposes.

Carbon nanotube applications

Many domains, such as optics, electronics, medicine and pharmaceutics, can benefit from the remarkable physicochemical parameters of CNTs, represented by high chemical stability, outstanding thermal and electrical properties. Furthermore, these high ordered molecules with a high length-to-diameter ratio can be used in drug delivery, antitumoral therapy, tissue repair, gene therapy, neurodegenerative diseases (Lacerda et al., 2006; Jain et al., 2012; He et al., 2013).

Firstly, before their utilization in the medical field, CNTs must be purified and functionalized. The functionalization process, which usually involves adding hydrophilic groups on the surface of biomaterials, is a required step in order to increase the solubility and biocompatibility of CNTs (Shim et al., 2002; Liu et al., 2009). Surfactants, such as Tween-20, Triton X-100 and sodium dodecyl sulfate, can be used for CNT functionalization, being stable only for an excess of surfactant. However, high concentration of surfactants can induce cell lysis and proteins' denaturation. Thus, the molecules used for coating should be biocompatible and very stable to withstand the detachment forces that can act when the functionalized CNTs are placed in aqueous solutions (particularly, in human serum due to high concentration of salts and proteins). Therefore, after functionalization, CNTs display binding surfaces for biomolecules and drugs, and the resulted complexes can be used in a large area of applications (Shim et al., 2002; Lacerda et al., 2006; Yang et al., 2007; Liu et al., 2009).

The most promising application of CNTs in medicine is represented by drug delivery, being used as transporters for various biological molecules. The conjugates CNT – therapeutic molecule can cross the cellular membrane through endocytosis or other mechanism, without inducing any toxic effect. Previously, the CNT surface was linked by functionalization with ligands that are recognized by cellular receptors. After internalization, the

caps are removed and the therapeutic molecule is released in the cell. It was proven that, the efficiency of delivery is better when the drugs are transported via CNTs, the biological molecules being kept intact without any participation to metabolic reactions during the transport (Bianco et al., 2005). All these results have influenced also the CNT application in cancer therapy. Several examples of drugs tested with CNTs are represented by epirubicin, doxorubicin, methotrexate, paclitaxel and quercetin (Bianco et al., 2005; He et al., 2013)

Since they have small dimensions, CNTs can pass through the blood-brain barrier and can be used as transporters in neurodegenerative disorders, including Alzheimer's disease. For example, SWCNTs can effectively be used to deliver acetylcholine in the brain of rats that mimic Alzheimer's disease. Other studies reported that carboxylated CNTs have antioxidant properties and can be used in the prevention of chronic diseases (Singh et al., 2012). SWCNTs have also application in gene therapy, where they can function as a cloning vector, without affecting transcription or translation processes (Yang et al., 2010; Liao et al., 2011; Singh et al., 2012).

In pharmacology, drugs are marketed as a mixture of enantiomers, out of which only one has therapeutic potential. It was proven that CNTs can be used in enantiomeric separation of these racemic mixtures (Silva et al., 2012). In the same time, as a consequence of electrical and mechanical properties, SWCNTs have found applications in improving some devices' performance, such as photovoltaic cells or batteries (Raffaelle et al., 2005; Silva et al., 2012).

CNTs-based detection systems have been proposed for measuring the expression of cancer biomarkers in early stage cancer diagnosis. By using novel methods based on CNTs with unique mechanical, thermal and electronic features, a high sensitivity detection can be achieved, with ultralow limits and wide linear ranges (Yu et al., 2006). The novel CNT-based approaches appear to be more efficient, in terms of detection, reliability, time and cost effectiveness, compared to conventional immunoassay tests, such as ELISA detection for prostate specific antigen, carcinoembryonic antigen, alpha-fetoprotein, carbohydrate antigen 19-9 and carcinoma antigen 125 (Ji et al., 2010). Furthermore, molecular imaging using CNTs as contrast medium in different traditional imaging technologies (computed tomography, magnetic resonance imaging and positron emission tomography) was developed recently providing a higher resolution (Chen et al., 2017). MWCNT`s hyper-echogenic nature makes it an excellent contrast agent for ultrasound imaging, which is a non-invasive, non-ionizing, real-time, cost-effective imaging technique. At the same time, biocompatible functionalized CNTs can be conjugated with anti-cancer drugs and tumour-targeting ligands (antibodies). Wu et al. (2014) conjugated shortened PEI-MWCNTs (polyethylenimine functionalized nanotubes) with fluorescein isothiocyanate (FITC) and prostate stem cell antigen (PSCA) monoclonal antibodies, thus constructing a novel nanoplatform which combines receptor-specific targeting, drug delivery, luminescence and ultrasound imaging into one system. Photoacoustic imaging of MWCNTs conjugated with Arg-Gly-Asp (RGD) peptides has also been used to detect mice bearing tumour xenografts. The authors speculate that SWCNT-RGD bind to the tumour vasculature from where they generate a consistent photoacoustic signal (De la Zerda et al. 2008).

Cytotoxic effects of carbon nanotubes

Recently, CNTs were intensively studied for their unique properties, which are responsible for their applications in various areas, including pharmacy and medicine. However, it is very important to assess the effects of these allotropes of carbon that can be induced at cellular and systemic levels (Cherukuri et al., 2004). One of the primary concerns when it comes to employing CNTs in biomedical applications is their potential toxicity, which can manifest in various forms, e.g. oxidative stress (Manke et al., 2013), fibrosis, upregulation of inflammatory pathways (Muller et al. 2005) and mutagenesis (Zhu et al., 2007).

The previous studies described three representative characteristics of particles known to induce cellular damage: (i) their high surface area which determines a good interaction with the cell membrane, (ii) the retention time, as they can interact a long time with the cell membrane, (iii) the toxicity and reactivity of substances' traces contained in nanoparticles (NPs) (Smart et al., 2006).

CNT toxicity is usually approached by evaluating the effects on cellular viability, as well as their capacity to induce the formation of reactive oxygen species (ROS) or mediators of inflammation. Further, functionalized CNTs were tested concomitantly with non-functionalized/raw CNTs to highlight the role of impurities (such as amorphous carbon and transition metals used in the synthesis of CNTs) and of functionalization in inducing cytotoxic effects (Sayes et al., 2006; Fiorito et al., 2006). Recent studies of intracellular uptake of CNTs indicated the existence of epigenetic alterations, alongside specific metabolic responses which could potentially be used to evaluate CNT biosafety (Chatterjee et al. 2017).

Raw/non-functionalized CNTs can induce ROS production in a dependent manner to impurities' concentration. The influence of CNT purification process on NR8383 (murine macrophages) and A549 (human lung cells) cultures was also reported previously. In this way, three types of carbon NPs were tested: SWCNTs with traces of impurities, purified MWCNTs and unpurified MWCNTs. The results showed an increase of ROS level at high concentrations of impurities, as a result of changes induced by CNTs on antioxidant systems after their cell uptake. All of these effects were mitigated when

the impurities were removed. The studies showed that the unpurified nanotubes induced oxidative stress which determined an increase of calcium concentration in the cytosol (Brown et al., 2004; Pulskamp et al., 2007). Other effects of ROS were correlated with the mitochondrial membrane potential changes. Interestingly, a reduction of mitochondrial membrane potential was noticed when NR8383 cells were exposed to CNTs, but this was not reported for A549 lung cells. These results suggest that the CNT particularities that appear to induce oxidative stress in cells are correlated with the nature and concentration of impurities as well as with the cell type used for exposure (Pulskamp et al., 2007).

Another report that focused on the effects of SWCNT on HEK293 cells showed a decrease of cellular viability and cell adhesion-related proteins in a time- and concentration-dependent manner. In other words, the expression of adhesion proteins (laminin, fibronectin and cadherin) was downregulated the more SWCNT concentration increased. Moreover, alterations associated with apoptosis (chromatin condensation, DNA fragmentation and apoptotic bodies formation) were detected after a 24 h incubation period with doses higher than 25 μg/mL CNTs (Cui et al., 2005).

Many studies have also focused their attention on the effects induced by SWCNTs on macrophages. Human and murine macrophages were exposed to purified SWCNTs, graphite NPs and fullerenes. The level of nitric oxide (a chemical mediator produced during inflammatory process), cellular viability and NPs' internalization were evaluated. Similar results were obtained when cells were exposed to SWCNTs and fullerenes. A low percentage of NPs was internalized which did not induce cell death or any inflammatory response. In contrast, graphite NPs were able to induce morphological changes, apoptosis and necrosis, and to increase nitric oxide level. These results show the graphite NPs are more cytotoxic than CNTs or fullerenes, most probably due to their intrinsic chemical structure, consisting of unpaired reactive bonds which can interact with biological molecules (Fiorito et al., 2006).

Having small sizes, CNTs can easily penetrate the epidermis and can be also inhaled. This readily uptake prompted the emergence of many risk assessment studies in order to predict the effects on workers routinely exposed to NP synthesis or other handling steps. Exposure of rats to doses higher than 5 mg/kg induced formation of granulomas at respiratory level, but the epidermis was not affected (Huczko and Lange 2001; Ema et al., 2011; Jain et al., 2012).

Conclusions

Due to their particular properties, CNTs show the potential to be ideal candidates for drug delivery, antitumoral therapy, tissue repair, gene therapy and neurodegenerative disease treatment. There are certain intrinsic parameters such as the purity, shape, size and surface chemistry (functionalization) of the nanotubes, which seem to highly influence a cytotoxicological response, thus limiting their biomedical use. However, the good biocompatibility of CNTs has facilitated their use in different *in vivo* studies with future prospective for clinical trials, which will have a great impact on developing new improved therapies.

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