Nanomedicine and Nanotoxicology

Ado Jorio *Editor*

Bioengineering Applications of Carbon Nanostructures

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Preface

Nanotechnology has been defined as the understanding, application and control of matter and processes at the nanoscale, dimensions typically between 1 and 100 nm. This is the range of biological processes and structures, thus placing nanoscience as the most important area for the development of biotechnology. New technologies for tissue engineering, intracellular transport, therapies, genetic engineering in animals and humans, the development of new vaccines and soil engineering has been dazzled thanks to nanotechnology.

Among nanomaterials, carbon nanostructures have attracted great attention from the scientific community over the past 40 years. The research initiated in the fields of carbon fibers, graphite intercalated compounds and amorphous carbons, generating a wide range of applications related to the unique electrical, thermal and mechanical properties of these materials. In sequence, the fullerenes were discovered and led to the Nobel Prize for chemistry in 1985; carbon nanotubes have generated an avalanche of studies in various areas of knowledge, giving rise to the Kavli Prize for Nanoscience in 2008; graphene launched the field of two-dimensional materials, leading to the Nobel Prize in Physics in 2010. With this avalanche came the mass production of carbon nanotubes and a huge variety of studies, including the noblest application of these nanomaterials, the biomedicine.

This book covers the development of different biomedical technologies based on carbon nanostructures. The focus is on carbon nanotubes, but fullerenes, amorphous carbons and graphene are also addressed. This work put together an interdisciplinary team from the *Research Center for Biotechnological Applications of Carbon Nanotubes*, including medical doctors, biologists, chemists and physicists working on biomedical applications of carbon nanostructures. They built this book that covers therapy, genetic engineering, tissue engineering, soils conditioning, toxicology and the basic aspects of synthesis and processing of carbon nanotubes for biomedical applications, in eight chapters.

The four first chapters are devoted to the use of carbon nanotubes in different biotechnology applications.

Chapter 1 discusses tissue engineering. Carbon nanotubes are among the unique materials that hold potential clinical applications in bone tissue engineering and orthopedic procedures, due to their capacity to accelerate bone regeneration. This chapter summarizes the recent developments in bone repair/regeneration using carbon nanotube composites, and provides insights on the future applications on bone tissue engineering.

Chapter 2 addresses genetic therapy using single wall carbon nanotubes as a delivery system for interfering RNA, to inhibit gene expression. Various strategies available and recent developments are discussed.

Chapter 3 discusses how carbon nanotubes can serve as DNA delivery agents for generation of genetically modified mammals embryos. Several research centers and pharmaceutical corporations use genetically modified animals in the development of new drugs, in the identification of new drug targets, and to test drugs' efficacy and safety. The use of carbon nanotubes as DNA deliver agents can be far simpler and less laborious when compared to other techniques to produce genetically modified mammals.

Chapter 4 verses about vaccines and the development of carbon nanotubes-based immunogens. The consistent use of vaccines is clearly the most cost-effective strategy, both at the individual patient level as well as a public health policy. However, classical vaccine strategies have been incapable to deliver satisfactory levels of immunogenicity and/or safety in several cases. This chapter considers the strategy of using functionalized carbon nanotubes as antigen carriers in vaccine formulations.

The next two chapters address new biotechnologies using other carbon nanostructures.

Chapter 5 discusses how fullerene-based materials can be used as therapeutic agents and contribute to reduce oxidative stress, with focus in the respiratory system diseases and neurodegenerative disorders. Oxidative stress is associated to the development and progression of several pathologies, including the neurodegenerative and pulmonary diseases.

Chapter 6 addresses the study of carbon nanostructures for soil fertility improvement and carbon storage. The work is built on the analysis of the carbon material found in an anthropogenic Amazonian soil that has been considered a potential model for organic matter soil storage, and for generating a sustainable land-use system that is highly efficient, even in the hot and humid tropical regions. Here the nanotechnology tools are being used to elucidate how nature solved the problem of keeping high levels of ion exchange capacity in these otherwise poor soils.

Finally, the two last chapters are devoted to general aspects that have impact on the development of biomedical applications.

Chapter 7 deals with the nanotoxicologic aspects of carbon-based nanomaterials. There are fundamental aspects that make usual toxicology procedures, for example related to chemicals, completely different from the nanotoxicology. In this sense, the carbon nanostructures appear as prototype models for the development of

nanotoxicology in general. The chapter covers results of in vitro and in vivo toxicological assessments of carbon nanostructures.

Chapter 8 is about the synthesis, purification and functionalization of carbon nanotubes, specifically for the biotechnological applications. Therefore, the book ends with what represents the initial steps researchers working in the biomedical applications have to give, which are their synthesis, purification and functionalization.

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Belo Horizonte, Brazil Ado Jorio
September 2014

Contents

Chapter 1
Bone Repair Utilizing Carbon Nanotubes

Paulo Antônio Martins-Júnior, Marcos Augusto de Sá, Vanessa Barbosa Andrade, Heder José Ribeiro and Anderson José Ferreira

Abstract One of the major challenges for researchers working on bone tissue engineering is developing biocomposites able to accelerate the repair of bone defects, thereby reducing the time and costs of patients rehabilitation. Since their discovery, carbon nanotubes (CNTs) have captivated investigators worldwide due to their remarkable mechanical, thermal and electrical properties, as well as their functionalization capability and biocompatibility. Recent studies have demonstrated that CNTs are among the unique biomaterials that hold potential clinical applications in bone tissue engineering and orthopedic procedures due to their impressive capacity of accelerating bone repair/regeneration. Significant progress has been achieved regarding the effects of CNTs associated or not with polymers in different experimental models (in vitro and in vivo). The purpose of this chapter is to summarize the recent developments in bone repair/regeneration using CNTs or CNT-based composites and to provide insights concerning future possible applications of CNTs on bone tissue engineering.

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1.1 Introduction

The bone tissue is the main component of the human skeletal system and, as such, it has a vital function in human daily lives [1]. Actually, there are many efforts to use nanotechnology, specially carbon nanotubes (CNTs), to emulate the natural nanostructure of the bone for orthopedic applications and for oral regenerative medicine. These include maxillofacial surgery and repair of cranial defects, as well as to support periodontal and implant regenerative procedures [1–4] (Fig. 1.1).

Currently, potential applications of CNTs in biochemical and biotechnological fields are immense due to their extraordinary structural, thermal, electrical and mechanical properties [5]. These properties make them a key element in nanotechnology since they can be used for the development of sensors and filter membranes, as well as in the creation of new biomaterials for tissue engineering [5–9].

This chapter provides essential information about the application of CNTs in the field of bone tissue engineering discussing selected literature. The chapter begins with a summary of the general properties of CNTs which allow this biomaterial to enhance bone repair/regeneration. Next, we will describe the constituents of the bone tissue, highlighting similarities between this tissue and CNTs. In the sequence, a compilation of recent and high-quality studies focused on the development of CNTs or CNT-based composites to be used in bone tissue engineering will be

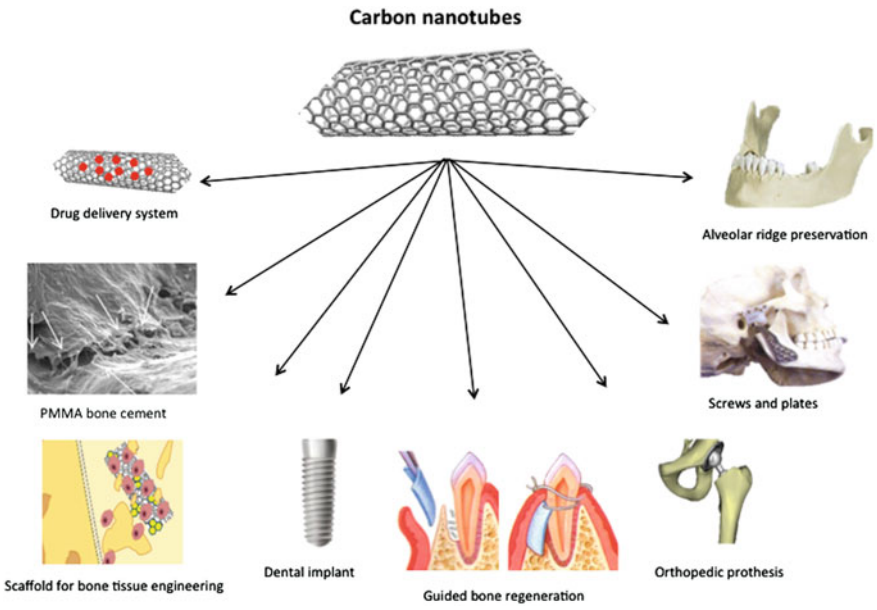

Fig. 1.1 Possible future applications of carbon nanotubes in bone tissue repair/regeneration engineering. *PMMA* polymethyl methacrylate

presented, describing particularities of each study to allow the understanding of the aims and main results achieved. Finally, we will give some insights into future possible applications of CNTs on bone tissue engineering.

1.2 General Properties of Carbon Nanotubes

CNTs have exceptional electronic properties directly related to their chirality and diameter. The similarity of the diameter between triple helix collagen and CNTs makes this nanocomposite an ideal material to promote bone growth [2, 10]. CNTs can present a high thermal and electrical conductivity [11]. Also, their electronic structure and ballistic conductance allows CNTs acting as biosensors and in the detection of biological molecules [12].

In addition to their electronic properties, CNTs have mechanical properties equally fascinating [12]. The strong covalent bond between carbon atoms has, as a consequence, high mechanical resistance, being one of the hardest and most resistant materials already known [13]. Because CNTs have low density and high mechanical strength as compared with bone and other materials used in bone implants, they can improve the mechanical properties of damaged bone tissue [10, 14].

Another important CNTs property that should be mentioned is the absence of dangling bonds, which gives this material great chemical inertness and reduced capacity of interaction with other materials, such as polymers. One of the main reasons is the poor dispersion of CNTs within water or organic solvents [15]. However, the functionalization process, i.e. their chemical association with different molecules, can improve their solubility and biocompatibility, reduce their toxicity and allow exceptional combination of the CNTs properties with those of other materials [2, 15–18]. For instance, the interaction between CNTs and polymers can result in biomaterials with increased capacity of inducing nucleation and growth of crystal hydroxyapatite [19] and osteogenesis [20].

The CNTs association with other materials, such as sodium hyaluronate (HY), can produce new composites with different electrical and mechanical properties. Indeed, it has been shown that the interaction between CNTs and HY (HY-CNT) results in a biocomposite with better biological and dynamic mechanical properties when compared with HY alone [15, 17].

1.3 Bone Tissue and Carbon Nanotubes

The bone tissue is a connective tissue formed by specialized cells and a mineralized extracellular matrix (ECM) impregnated of calcium phosphate organized in hydroxyapatite crystals. Although there are at least 28 types of collagen, the non-mineralized component of the bone ECM is composed by types I, III, V, XI

Table 1.1 Types of collagen present in the non-mineralized component of the bone extracellular matrix and their distribution throughout the body

Type	Collagen family	Tissue distribution
I	Fibril-forming collagens	Bone, dermis, tendon, ligaments and cornea
III	Fibril-forming collagens	Skin, vessel wall and reticular fibers of most tissues (lungs, liver, spleen, etc.)
V	Fibril-forming collagens	Lung, cornea, bone, fetal membranes and together with type I collagen
XI	Fibril-forming collagens	Cartilage and vitreous body
XIII	Transmembrane collagens	Epidermis, hair follicle, endomysium, intestine, chondrocytes, lungs and liver

and XIII collagens [21, 22] (Table 1.1). Other molecules are also presented in the organic ECM of the bone tissue and they include proteoglycans and glycosaminoglycans [22, 23]. The interaction between inorganic and organic ECM components provides structure strength and resistance to the bone. Interestingly, CNTs can act as nucleation sites during the deposition of the bone matrix, inducing crystal nucleation and growth of the inorganic component of the bone [24].

The osteoblasts (Fig. 1.2) are the cells responsible for synthetizing bone matrix organic components such as type I collagen, proteoglycans and glycoproteins. Also, they synthetize proteins essential to calcium binding, i.e. osteopontin, osteocalcin, sialoprotein I and II, and receptor activator of nuclear factor kappa B ligand (RANKL) [25]. It has been reported that the biocomposite formed by the association of CNTs with HY induces an important stimulatory effect on the expression of bone morphogenetic protein-2 (BMP-2) and osteopontin, which likely is involved in the accelerating action in the bone healing process triggered by this biocomposite [17]. Additionally, recent studies have shown that CNTs sustain the growth of osteoblasts and bone formation, allowing the cells to adhere, proliferate and secrete bone matrix in an earlier stage [26].

Another cell type present in the bone matrix is the osteoclasts (Fig. 1.2). They are derived from circulating monocytes, which merge into the bone matrix forming a multinucleated cell. The osteoclasts are found in resorption areas of the bone matrix. The formation of osteoclasts is regulated by osteoblasts through the production of colony stimulating factor monocytes (M-CSF), which induces the differentiation of monocytes into osteoclastic precursor cells [27]. Importantly, Narita et al. [28] reported that CNTs could act inhibiting osteoclastic bone resorption, suggesting that they have crucial effects on bone remodelling.

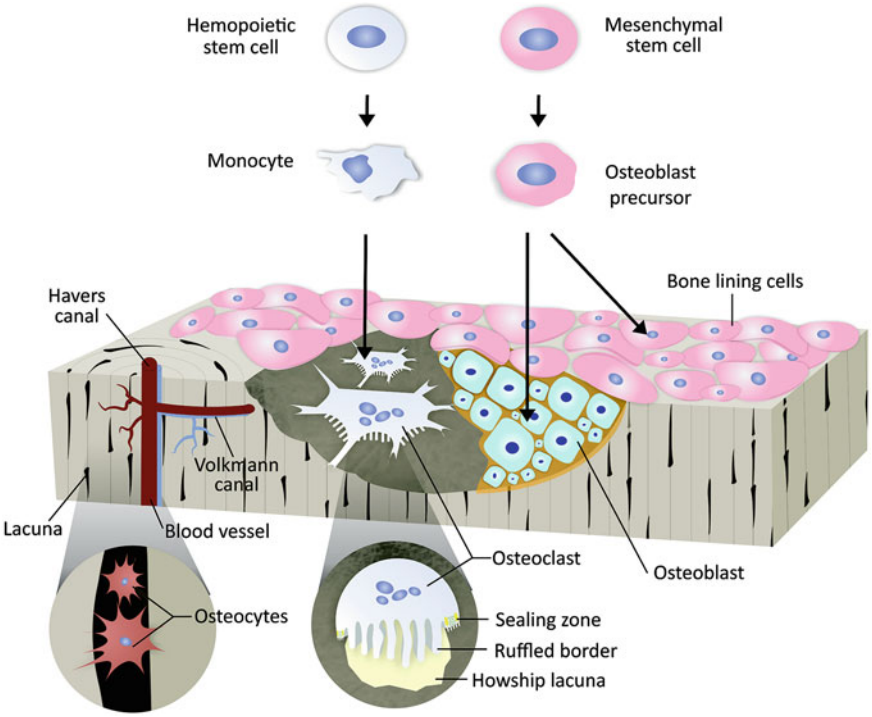

Fig. 1.2 Cytoarchitecture of the bone tissue. This schematic figure shows the differentiation of hemopoietic stem cells into monocytes and their subsequent differentiation into osteoclasts, which are multinucleated giant cells responsible for resorption of the bone matrix. This process occurs by adhesion of osteoclasts to the matrix through the sealing zone, forming a microenvironment called howship lacuna. The ruffled border is the active zone of the osteoclasts with villous surface shape and in this region acid (H^+) and enzymes responsible for the degradation of the organic matrix are released. Moreover, we can observe the differentiation of mesenchymal stem cells into osteoblast precursor cells that subsequently differentiate into osteoblasts, which are cells responsible for the synthesis of bone matrix. In the bone matrix there are lacunas, and osteocytes are located into these lacunas. Osteocytes are cells fully involved by bone matrix and have oval body with extensions. It is also observed in the lower left part of the scheme, the organization of secondary bone tissue in lamellae concentrically arranged forming the Havers' systems. The Havers' systems are made by Havers' channels, i.e. central structure that houses the blood vessels responsible for irrigation of the bone tissue. Communication between two Havers' channels is taken by Volkmann's channels

1.4 Application of Carbon Nanotubes in Bone Repair

One of the major challenges for researchers working on bone tissue engineering is to develop biocomposites which are able to accelerate the repair of bone defects, thereby reducing the time and costs of patients rehabilitation. Indeed, advances in the nanotechnology knowledge have generated great interest in the field of

orthopedic rehabilitation and in various areas of dentistry, such as implantology, periodontics and maxillofacial surgery [2].

In recent years, significant progress was achieved regarding the effects of CNTs associated or not with polymers in different experimental models (in vitro and in vivo) [17, 18, 26, 29, 30] (Table 1.2).

For instance, the characteristics of single wall carbon nanotubes (SWCNTs) and SWCNTs associated with HY (HY-SWCNTs) have been assessed using Raman spectroscopy (Fig. 1.3) [17, 18]. The Raman spectra of these biocomposites showed relatively low ratio between D and G bands, which means relatively low amount of structural defects and amorphous carbon. Furthermore, the presence of the radial breathing mode (RBM) characterized these nanotubes as SWCNTs. The absence of major differences between the Raman spectra of the SWCNTs and HY-SWCNTs showed a non-covalent association between HY and SWCNTs (Fig. 1.3) [18]. Therefore, association of HY with SWCNTs results in a more reinforced and stable gel, which is essential for biological applications [15, 17].

Then, Mendes et al. [17] evaluated the effects of HY-SWCNT on bone repair of tooth sockets of rats. The sockets treated with the nanocomposite had a higher percentage of newly formed bone trabeculae when compared with control sockets. Furthermore, the expression of type I collagen was also higher in sockets treated with HY-SWCNTs [17].

Afterwards, Sá et al. [18] investigated the effects of HY-SWCNTs in conditions where bone healing is hindered. It is well established that diabetes alters bone metabolism, reducing its formation and resorption, prolonging the process of bone tissue repair [31]. Thus, the effects of HY-SWCNTs were tested on bone repair of tooth sockets of rats with type I diabetes [18]. The results showed that 14 days after the extractions, the percentage of newly formed bone trabeculae was significantly higher in tooth sockets of diabetic rats treated with the nanocomposite. The treatment increased the bone formation and also markedly reduced the number of cell nuclei, reaching values similar to those observed in sockets of non-diabetic animals. Therefore, treatment with HY-SWCNTs was able to restore the bone repair process in tooth sockets of diabetic rats [18].

The above-mentioned studies demonstrated the great potential of HY-SWCNTs in future dental clinical applications [17, 18]. In addition, the association of CNTs with HY generated a nanocomposite with stronger and more rigid structure than HY native gels, suggesting that this combination produces a more resistant and stable material [15]. CNTs are capable of stabilizing the HY in their walls, which can be measured by thermogravimetric analysis (Fig. 1.4). Altogether, apparently, the use of HY-SWCNTs is better than HY alone, since CNTs improve the viscoelastic properties of the HY, while preserve its biological effects [15, 17, 18].

Several in vitro and in vivo studies investigated the effects of CNTs combined with other polymers on bone pathophysiology [26, 29, 30, 32–35]. Tutak et al. [26] evaluated the effects of SWCNTs associated with a multicelulose ester membrane on the osteoblastic cells behaviour and observed an initial toxicity after 24 h of exposure to the composite, leading to a reduction in the cell viability. This result was attributed to an increase in the inclusion of CNTs internalized by the cells.

Table 1.2 Recent studies using carbon nanotubes for bone tissue engineering purposes

Authors/year	Study design	Biomaterial	Main findings
Mendes et al. [17]	In vivo	HY-SWCNTs	Higher percentage of bone formation and higher expression of type I collagen in treated sockets
Sá et al. 2013 [18]	In vivo	HY-SWCNTs	Higher percentage of bone formation and reduction in the number of cell nuclei in sockets of diabetic rats
Tutak et al. [26]	In vitro	SWCNTs on MCE	Initial toxicity related to the inclusion of SWCNTs by osteoblasts; cell viability gradually increased
Abarrategi et al. [29]	In vitro and in vivo	MWCNT-CHI composite	Suitable biocompatibility; preservation of the osteogenic differentiation activity on MWCNT-CHI and ectopic bone formation
Shimizu et al. [30]	In vitro	MWCNTs and MWCNT-Collagen-rhBMP-2 composite	Higher ectopic bone formation; higher degree of calcification; higher expression of osteocalcin and higher amount of calcium in osteoblasts cell culture treated with MWCNTs.
Liao et al. [32]	In vitro	PP-MWCNT-nHA nanocomposite	MWCNTs enhanced the stiffness, tensile strength and impact toughness of the nanocomposite; increased proliferation of osteoblasts
Pan et al. 2012 [33]	In vitro	MWCNT-polycaprolactone	Improved mechanical properties of composite scaffolds by MWCNTs; differentiation of BMSCs on osteogenic lineage and high ALP activity
Cheng et al. [34]	In vitro	CNT-PLGA	Higher mechanical strength; increased attachment and proliferation of osteoblasts and higher rate of differentiation
Gupta et al. [35]	In vitro	SWCNTs, PLGA and SWCNT-PLGA composite	Biocompatible; composites containing SWCNTs increased cell proliferation and expression of bone repair markers

Abbreviations: *ALP* Alkaline phosphatase; *BMSCs* Bone marrow-derived stromal cells; *CHI* Chitosan; *CNTs* Carbon nanotubes; *HY* Sodium hyaluronate; *MCE* Multicellulose ester membrane; *MWCNTs* Multi-walled carbon nanotubes; *nHA* Hydroxyapatite nanorods; *PLGA* Poly (lactide-co-glycolide); *PP* Polypropylene; *rhBMP-2* Recombinant human bone morphogenetic protein-2; *SWCNTs* Single-walled carbon nanotubes

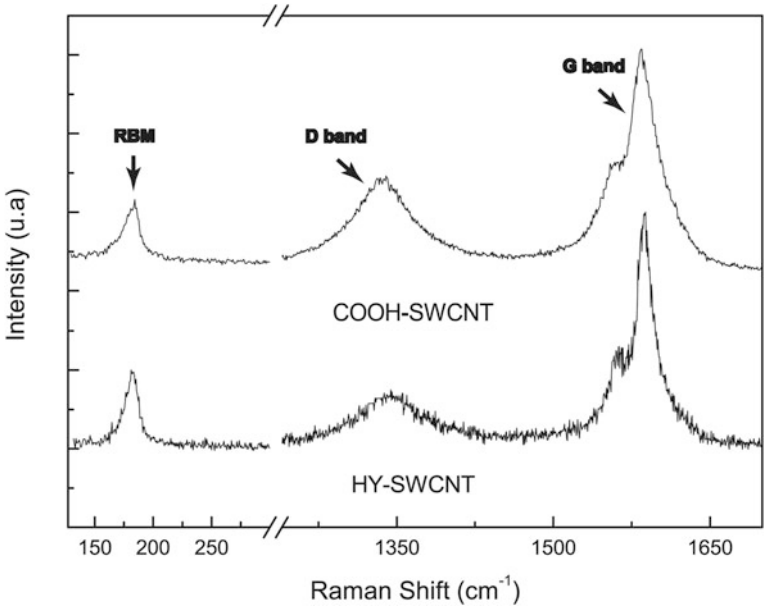

Fig. 1.3 Raman spectroscopy of SWCNTs and HY-SWCNT. Spectra are showing the radial breathing mode (*RBM*), D and G bands. The low ratio between D and G bands indicates that SWCNTs presented relatively low amount of structural defects and amorphous carbon. The similarity of the SWCNTs and HY-SWCNTs spectrum suggests a non-covalent association between HY and SWCNTs. Reproduced from Ref. [18]

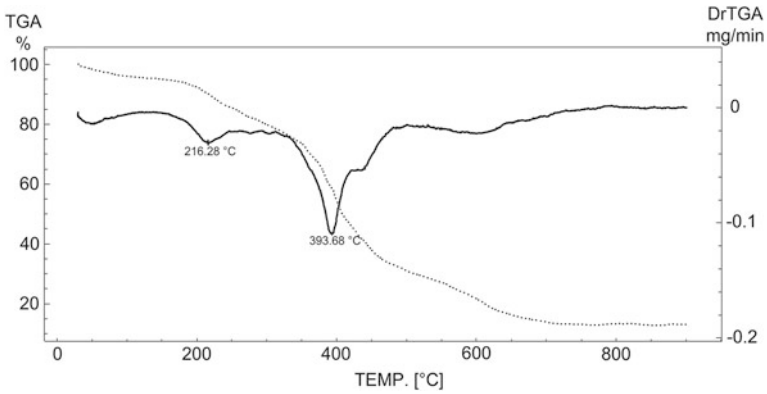

Fig. 1.4 Thermogravimetry (TG) curve of the burning of HY-SWCNTs. It is possible to observe two declines in the percentage of mass (*dotted line*) and, more specifically, the initial and final temperatures of the material burning (*continuous line*). The first reduction of mass happened at ~216.28 °C and represents the burning of the HY. The burning of the SWCNTs occurred at ~393.68 °C (*second drop*)

However, in successive time points evaluated, the cell viability progressively increased in cells treated with the nanocomposite. These later results were attributed to an increased expression of type I collagen, as well as to the release in the culture medium of protein markers of bone repair from the early dead cells [26].

A recent study evaluated a polypropylene biocomposite reinforced with multi walled CNTs (MWCNTs) and hydroxyapatite nanorods (PP-MWCNT-nHA) [32]. The authors found that the incorporation of MWCNTs was beneficial not only by enhancing the stiffness, tensile strength and impact toughness of the nanocomposite, but also by increasing the proliferation of the osteoblasts, especially after 7 days of the treatment. This later result can be explained by the fact that the nanometric dimensions of the biomaterial acted like an efficient seeding site for osteoblasts. Cheng et al. [34] developed a porous scaffold composed of CNTs and poly(lactide-co-glycolide) (PLGA) with appropriate mechanical strength and controllable surface roughness for bone repair. They found that the incorporation of CNTs into PLGA resulted in higher mechanical strength when compared with scaffolds containing only PLGA. Additionally, such incorporation led to an increased attachment and proliferation of osteoblasts and a higher rate of differentiation. Similarly, Gupta et al. [35] developed a SWCNT-PLGA composite for orthopedic applications and evaluated their interaction with human stem cells and osteoblasts. Imaging studies revealed that both osteoblasts and stem cells exhibited normal and non-stressed morphology and the material was biocompatible. Moreover, the composite increased cell proliferation and gene expression of bone markers (alkaline phosphatase, type I collagen, osteocalcin, osteopontin, Runx-2 and bone sialoprotein). The authors concluded that the SWCNT/PLGA composite had beneficial effects on cellular growth and gene expression of bone markers.

MWCNT-polycaprolactone composite scaffolds were fabricated and tested for bone tissue engineering applications [33]. It was observed that mechanical properties of the composite scaffolds were improved by the addition of MWCNTs. Rat bone marrow-derived stromal cells (BMSCs) seeded in the scaffold differentiated down into osteogenic lineage and showed high activity of alkaline phosphatase. Interestingly, the authors found that the scaffolds with low concentration of MWCNTs enhanced the proliferation and differentiation of BMSCs more than that with higher concentration of MWCNTs.

Abarretagi et al. [29] studied the behaviour of myoblast cells in the presence of a biomaterial composed by MWCNTs and chitosan (CHI) in small quantities (MWCNT-CHI) [29]. This biomaterial did not affect the cell viability. Combining MWCNT-CHI with recombinant human bone morphogenic protein-2 (rhBMP2) caused osteogenic differentiation in vitro and ectopic bone formation in vivo when it was implanted in a subcutaneous pocket in the dorsal muscle of mice. Apparently, MWCNT-CHI was progressively degraded without evidence of cytotoxic effects [29]. Similarly, implants composed by collagen, MWCNTs and rhBMP2 inserted in the dorsal region of mice also caused a significant ectopic bone formation [30]. In addition, primary osteoblasts incubated with MWCNTs presented a significant increase in the deposition of mineralized nodules, with no changes in cell viability and increased expression of osteocalcin mRNA [30]. Together, all these data

demonstrate that CNTs increase the bone formation without any significant cytotoxic effect. However, the molecular mechanisms by which CNTs induce these actions are not fully understood and further studies are needed in order to clarify this issue.

1.5 Perspectives on Carbon Nanotubes in Bone Repair

It is well established that orthopedic and maxillofacial surgeons often employ orthopedic implants/biomaterials to deal with large bone defects caused by trauma, congenital anomaly, tumour resection or musculoskeletal diseases [34]. Most of orthopedic implants/biomaterials are generally made of metals (including titanium), ceramics, hard polymers or their composites, or they are scaffolds for tissue regeneration, which are based on polymers [4, 32]. However, limitations in the structural and biological compatibility of these materials with natural bone tissue can lead to bone loss from the implantation site and subsequent loosening of the implants, implant failures and complicated revision surgeries [36–38]. When compared to titanium, CNTs are physically stronger [39], lighter [10] and they have excellent flexibility, as well as a potential to form surfaces able of mimic the ECM morphology with the capability of facilitating the hydroxyapatite crystallization [40].

Due to their excellent aforementioned physical properties, CNTs can serve as a mechanical reinforcement for polymers and ceramic composites and form nanostructured coatings to enhance the bioactivity of implant surfaces, acting as scaffolds to promote and guide bone tissue regeneration [4, 14, 37]. CNTs are lightweight, strong and can be formulated in a manner to mimic the initial nanostructures of the bone components, such as hydroxyapatite and collagen fibers [14, 41].

Therefore, the inclusion of CNTs on the composition of biomaterials applied to the bone, such as high-strength arthroplasty prostheses, fixation plates and screws, offers a great possibility to improve the overall mechanical properties of these materials and to accelerate osseointegration [1, 42]. An example of the usage of CNTs in orthopedic applications is the reinforcement of the hydroxyapatite structure by CNTs. Hydroxyapatite has been extensively used for maxillofacial surgery, orthopedics and implant fabrication [43–45]. However, the poor mechanical properties of the hydroxyapatite regarding to brittleness and low fracture toughness restrict its use in load bearing applications (orthopedic/dental implant) [45, 46]. Thus, the addition of CNTs to the hydroxyapatite structure could create a more resistant material with enhanced biocompatibility.

Previous studies have shown that the addition of MWCNTs to polymethyl methacrylate (PMMA) significantly improves its static properties and fatigue performance [47–49]. PMMA is the primary component of acrylic bone cements and it has been reported that active or overweight patients with implants fixed with PMMA bone cements are at risk of cement mantle failure [48]. Thus, the incorporation of CNTs into polymers could be used to improve their mechanical, thermal and electrical properties, while retaining the structural capabilities of the polymer matrix. Besides that, including MWCNTs into implants for the treatment of bone

fractures, such as plates and screws, might promote bone repair and facilitate rapid fracture healing [42].

Alumina ceramics are widely used in orthopedic surgery [50, 51]. Also, they are routinely utilized in components of knee and ankle joint prostheses, as artificial bone to repair cranial and orbital bone defects and for dental and cochlear implants [45, 51–57]. Nonetheless, ceramics possess low strength and great risk of damage [58, 59]. Recently, Ogihara et al. [51] added CNTs as reinforcement for an alumina ceramic base material and they achieved remarkable improvements in the fracture toughness along with good biocompatibility and bone tissue compatibility. Therefore, it is expected that CNTs/alumina composites become an alternative biomaterial for development of prostheses for arthroplasty and bone repair.

Another possibility is to take advantage of the highly textured surface of the CNTs, since this is expected to influence osteoblast behaviour. The exceptional nanotopography of the CNTs surfaces provides enhanced osteoblastic adhesion onto the surface matrices due to the higher adsorption of fibronectin and vibronectin proteins, thereby improving the cell-surface interactions [32, 60–62]. Furthermore, nanostructured surfaces may be capable of controlling stem cell fate. Indeed, a CNTs-covered surface could stimulate osteoblast cells to re-synthesize the mineralized matrix and allow mesenchymal stem cells to attach and differentiate to deliver a mature population of osteoblasts for osseointegration and rapid bone regeneration [63].

The chemical and electrical properties of CNTs can also be explored for future advances in bone implant materials [37]. CNTs could be used as scaffolds to promote and guide bone-tissue regeneration [10, 24, 42] or as local drug-delivery systems (DDS) to distribute molecular stimulants for cell adhesion, growth and proliferation, as well as drug loads to control immunological attack to the new bone implants [37]. In addition, CNTs could help developing smart multifunctional orthopedic implants with the capacity for monitoring implant interactions with ECM components, as well as to act like substrates for osteoblast cell growth due to their chemical and electrical conductivity [37].

In oral regenerative medicine, bone reconstruction is often an essential requirement for functional rehabilitation of the stomatognathic system [2]. In this way, CNTs could be used as a potential biomaterial to help oral maxillofacial surgeons in achieving successful outcomes in a wide range of regenerative procedures. For example, severe loss of attachment level and deficient alveolar sites caused by periodontal disease could be treated by a combination of guided bone regeneration (GBR) [64] and membranes composed by CNTs. The unique porous 3D structure of the CNTs combined to reconstructive periodontal technology would create a more predicable environment to different tissues (gingival, cementum, alveolar bone and periodontal connective tissue) integration and proliferation [2]. Another possibility is the direct application of CNTs in fresh tooth sockets (covered or not by a resorbable membrane) aiming to allow better alveolar ridge preservation. Also, CNTs may be used alone or in combination with other polymers into receptor site of dental implants to accelerate the osseointegration process, thereby reducing the postoperative and the oral rehabilitation period of the patients [2].

1.6 Conclusion and Perspectives

Since their discovery, CNTs have captivated researches worldwide due to their remarkable mechanical, thermal and electrical properties, as well as their functionalization capability and biocompatibility. Recent studies have demonstrated that CNTs are among the unique biomaterials that hold potential clinical applications in bone tissue engineering and orthopedic procedures due to their impressive capacity of accelerating bone repair/regeneration. However, several points regarding their mechanisms of action, modulation of inflammatory process and toxicology are not well understood. Therefore, the aim of future researches should be to establish the safety of CNTs for human use before their incorporation into clinical applications for bone repair/regeneration.

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Chapter 2
The Use of Single Wall Carbon Nanotubes as a Delivery System for siRNA

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Abstract RNA interfering (RNAi) is a biological process that operates in most eukaryotic cells in which small interfering RNA (siRNA) molecules inhibit gene expression. Recently, RNAi has been recognized as a therapeutic option to treat several diseases. However, the use of this gene silence technology has been limited, mainly because of the low efficiency of the different vectors in delivering significant amounts of siRNA to cells. In this context, CNTs have emerged as a novel vector to deliver siRNA for post transcriptional gene silencing purposes in vitro and in vivo due to their unique chemical and physical properties. This chapter is focused on the various strategies available and recent developments on the applications of Single Wall Carbon Nanotubes (SWCNTs) as a nanovector for siRNA delivery.

2.1 Introduction

2.1.1 CNTs as Molecular Transporters

Carbon nanotubes (CNTs) are fullerene-related structures consisting of hexagonal arrangement of sp^2 hybridized carbon atoms. Nanotubes can have a single layer of

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graphene sheets (single-walled CNTs) or can be made of multiple layers (multi-walled CNTs), and both have been explored for biological utility. When a nanotube contains only two layers, it is referenced as double-walled carbon nanotube (DWCNT).

As a molecular transporter, CNTs can shuttle various types of molecules into cells, among them proteins [1, 2], DNA [3], RNA (for references see bellow), and commonly used drugs [4–6]. In this regard, previous work from several laboratories involving different cell types have demonstrated the uptake of various types of functionalized CNTs, with most studies supporting the fact that CNTs enter cells via endocytosis [7, 8]. The endocytosis pathway is an energy-dependent process in which cells can engulf different molecules. In addition, it has been proposed that CNTs can penetrate the cells through passive-diffusion pathway in a needle-like manner [3]. However, this endocytosis-independent cell entry mechanism is still controversial, and more studies need to be performed in order to fully understand the mechanisms involved in CNT uptake by the cells.

2.1.2 Application of CNTs as a Delivery System for siRNA

Gene therapy has been recognized as a potential tool to treat several disorders. Among the various types of gene therapy available, RNA interfering (RNAi) represents a novel therapeutic approach for treating severe and chronic diseases due to its high specificity [9]. RNAi, also called post transcriptional gene silencing (PTGS), is a naturally occurring process in which a target gene is silenced. For successful knocking down of genes by RNAi, an efficient delivery system is required to allow proper membrane penetration, low immunogenicity, and protection from degradation [10]. In this regard, a variety of delivery agents have been applied to overcome these problems, but with limited degree of success [10]. In the last years, CNTs have emerged as a novel and alternative delivery agent for different biomolecules, including small interfering RNA (siRNA). In fact, CNTs not only carry the attached molecules into cells, but also present low cytotoxic effects into different types of cells [11]. In addition, CNTs provide protection to the cargo from degradation while in the circulation [11]. However, an important disadvantage related to the CNTs is its lack of solubility, and this drawback is mostly solved by incorporation of different pendant units to CNTs. Thus, functionalization of CNTs is very important for most of their use in biological applications, providing several advantages, including enhanced solubility in water, increased dispersion, and a lower tendency to form agglomerates [12]. In addition, functionalization increases the SWCNT half-life in the plasma, as demonstrated by Kirkpatrick et al. who showed that polyethylene glycol (PEG)-lipid solution in the formulation of the SWCNT complex prolonged its blood circulation time from minutes to hours [11]. In viewpoint of functionalization type, many kinds of strategies have been explored for siRNA delivery, and among these we can cite: (i) noncovalent functionalization which include the use of many biomolecules, polymers, and surfactants, and (ii) covalent functionalization that include chemical modifications [12].

This chapter summarizes the various strategies available for CNT functionalization and recent developments on the applications of Single Wall Carbon Nanotubes (SWCNTs) as a nanovector for siRNA delivery in vitro (Sect. 2.2) and in vivo (Sect. 2.3), with special focus on the hard-to-transfect cells, such as cardiomyocytes (Sect. 2.4). Section 2.5 gives a brief summary and discusses the perspectives for the use of CNTs for siRNA delivery.

2.2 Strategies Available for SWCNT Functionalization and siRNA Delivery In Vitro

Several functionalization strategies that include covalent modification or noncovalent approaches are currently available to enable biological application of CNTs. Polymers such as polyethylene glycol (PEG) and PEGylated phospholipids are known for their high biocompatibility and dispersibility, thus making them efficient surface enhancers for CNTs. Kam et al. [13] were one of the first to report the conjugation of SWCNTs to siRNA, and their use as nanocarriers. Their strategy involved the use of phospholipids functionalized SWCNTs through cleavable disulfide linkage to enable controlled siRNA release from the nanotube surface. This approach demonstrated to be highly efficient in delivering siRNA to HeLa cells and more potent than a commercially available transfection agent. The superior efficiency of these functionalized nanotubes in gene silencing was attributed to efficient siRNA cargo loading, and to the intracellular cleavable disulfide links that facilitates the endosome/lysosome escape of siRNA to the cytosol, protecting it from degradation. Extending these findings, Liu et al. [14] functionalized SWCNTs by adsorption of phospholipids grafted onto amino-terminated PEG. The complex siRNA:SWCNTs was then attached by disulfide bonds, as previously described by Kam et al. [13] and applied to human T cells and primary cells. By using this technique the authors once again demonstrated that SWCNTs can be effectively used as transporters for siRNA to silence the targeting gene on different cell types with superior results over conventional nonviral approaches, such as liposomes.

By using a different strategy, Yang and coworkers [15] investigated the ability of phagocytic cells to engulf siRNA complexed with positively charged SWCNTs (SWCNT+). Consistent with the idea that endocytosis and phagocytosis are important mechanisms of penetration of SWCNTs in the cells, siRNA:SWCNT+ complex was efficiently taken up by phagocytic dendritic cells. In addition, SWCNTs carrying CD80 targeted siRNA when incubated with dendritic cells led to significant reduction in CD80 expression in these cells, without affecting transcript expression of a housekeeping gene.

The use of siRNA has been shown efficient in the treatment of many diseases including various cancers [10]. Zhang et al. [16] explored the use of positively charged functionalized SWCNTs that were complexed to siRNA targeted to telomerase reverse transcriptase (TERT) and incubated with tumor cells. Telomerase is a cellular ribonucleoprotein found in eukariotic cells that maintains the tandem

telomeric TTAGGG repeats at chromosome ends [17], and its activation is critical for immortalization. TERT is its proteinaceous catalytic subunit which plays a critical role in tumor development and growth through the maintenance of telomere structure. Corroborating previous findings, the complex TERT siRNA:SWCNTs+ was efficient in delivering siRNA and knockdown the expression of TERT in a variety of tumor cells. In addition, TERT siRNA:SWCNT+ complex significantly suppressed the growth of LLC, TC-1, and 1H8 tumor cells and reduced cell number after 6 days of incubation with the cells [16]. Another attempt to investigate the potential of siRNA:SWCNT complex to prevent tumor progression was performed by Wang et al. [18], who employed ammonium-functionalized SWCNTs electrostatically bound to siRNA targeting cyclin A2. Cyclin A2 is a key regulator of cell cycle, and its overexpression has been detected and related to many types of cancers. The authors successfully reported the suppression of cyclin A2 expression in a tumour cell line (human erythroleukemic cell line, K526) by using the complex cyclin A2 siRNA: CNT. Moreover, it was observed growth inhibition and apoptosis of the cells incubated with the complex. Further in 2012, Chen et al. [19] also demonstrated that chemically functionalized SWCNTs can delivery siRNA to cancer cells. Accordingly, the authors used siRNA targeting MDM2 (murine double Minute clone 2) which was linked to functionalized SWCNTs by using DSPE-PEG2000-Amine, and the complex applied to breast cancer cells (carcinoma B-Cap-37). MDM2 is known to inactivate the tumor suppressor protein p53. The results indicated that siRNA-MDM2-SWCNTs complex was efficiently taken up by the cells leading to proliferation inhibition and apoptosis of the cells. Taking together, these studies confirm the efficiency of SWCNTs as nanovectors for siRNA delivery in a variety of tumor cells supporting its potential use for in vivo therapies.

The time dependence of CNTs as nanocarriers was evaluated by our group in 2010 using SKHep1 cells [20]. SKHep1 cells are a liver-derived epithelial cell line, capable of proliferating, that are not polarized. In this work, we used short

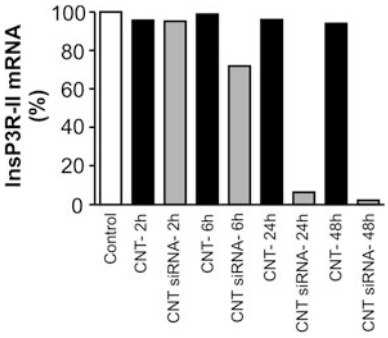

Fig. 2.1 Time dependence of SWCNTs as siRNA carriers in SKHep1 cells. Cells were incubated with the complex InsP3R-II siRNA:CNT for 2, 6, 24 and 48 h, after which media was replaced by regular culture media. Control group was exposed to a suspension of carboxylated SWCNTs alone for the time period indicated in the graph. InsP3R-II mRNA levels were examined 48 h after incubation began. Significant InsP3R-II gene knockdown was observed in cells incubated with the complex for 24 and 48 h. Reproduced from Ladeira *et al.* with permission from Nanotechnology [20]

single-wall (approximate length 200 nm) carboxylic-functionalized CNTs, and siRNA targeted to InsP3R-II, an intracellular Ca^{2+} channel present in the endoplasmic reticulum of SKHep1 cells. In order to determine the optimal time that provides maximum gene knockdown, we exposed cells to InsP3R-II siRNA: SWCNT complex for 2, 6, 24 and 48 h, and we performed quantitative real-time PCR experiments to assess levels of InsP3R-II mRNA. As shown in Fig. 2.1, a reduction in InsP3R-II mRNA levels was observed 6 h after the incubation with CNT-siRNA complex. However, higher InsP3R-II gene knockdown was achieved when cells were exposed to the complex for longer periods of time (24 and 48 h). In addition, cells incubated with CNTs alone did not show any alteration in InsP3R-II mRNA levels (Fig. 2.1). Likewise, Neagoe et al. in 2012 [21] used carboxylated

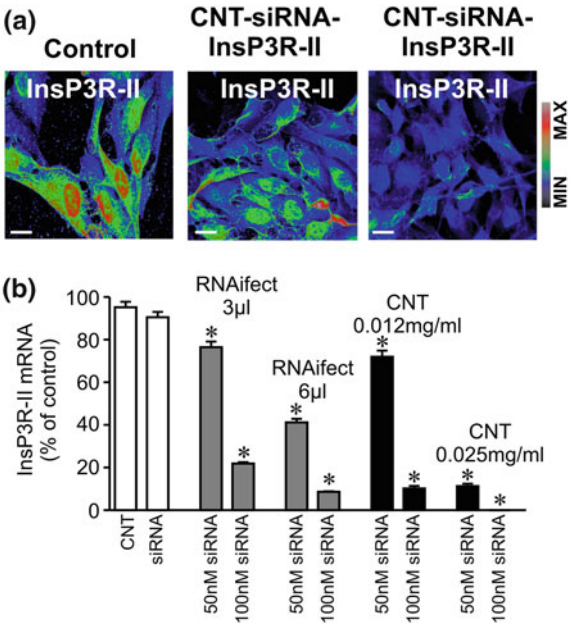

Fig. 2.2 InsP3R-II siRNA:SWCNT complex reduces InsP3R-II expression levels in SKHep1 cells. **a** Representative confocal images showing InsP3R-II labeled cells. InsP3R-II staining was significantly reduced in SKHep1 cells transfected with InsP3R-II siRNA:CNT (*middle* and *right panels*) when compared to control cells (*left panel*). Confocal images were collected 48 h after transfection began. Final concentrations of 50 nM *(middle panel)* or 100 nM siRNA *(right panel)* were efficiently delivered by SWCNTs in SKHep 1 cells. Images were pseudocolored according to the gray scale (color online). Scale bar = 10 µm. **b** Real-time PCR comparing type II InsP3R mRNA expression levels following siRNA delivery using SWCNTs or a lipid-based gene transfer system as carriers. siRNA:RNAifect reagent combination was evaluated at ratios of 50 nM:3 µL, 100 nM:3 µL, 50 nM:6 µL and 100 nM:6 µL. For siRNA:SWCNT complex formation different concentrations of SWCNTs (0.012 or 0.025 mg/mL) were added to the diluted siRNA (50 or 100 nM final concentration). Both delivery agents were efficient in suppressing InsP3R-II gene expression in SKHep1 cells. * = $p < 0.05$ when compared with control group. Reproduced from Ladeira et al. with permission from Nanotechnology [20]

SWCNTs as nanocarriers of siRNA, and by using this strategy these authors obtained efficient siRNA delivery and potent gene silencing in Hep2G cells.

In addition, we observed a concentration dependence on silencing efficiency, since a higher SWCNT to siRNA ratio produced more efficient InsP3-RII knocking down, as shown in Fig. 2.2. This finding is consistent with the idea proposed by Cherukuri et al. [22] that CNT uptake into the cells increases as a function of CNT concentration in the medium. Like previous studies, SWCNTs demonstrated superior silencing efficiency over a conventional lipid based-transfection agent (Fig. 2.2b).

We also investigated the effects of SWCNTs on SKHep1 viability by performing FACS (Fluorescence Activated Cell Sorting) in Anexin-V–FITC and propidium iodide (PI)-stained cells exposed to the same concentration of SWCNTs used in the siRNA experiments. As shown in Fig. 2.3, no effect of SWCNTs on SKHep 1 cell viability was observed indicating that under the conditions used for RNAi, CNTs do not interfere with SKHep1 functionality. These data correlated with findings from a previous study [23] showing that the uptake of SWCNTs did not adversely affect HL60 cells at similar CNT concentration.

Thus based on the literature, SWCNTs are considered to be effective carriers of siRNA in a variety of cells, regardless of the amount and type of functionalization used in the SWCNT surface.

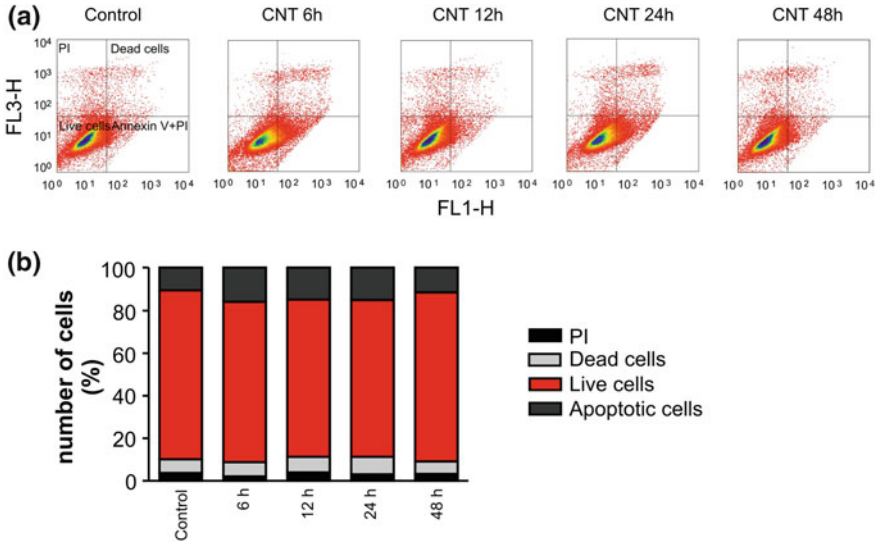

Fig. 2.3 CNTs do not affect SKHep 1 cell viability. Cellular viability was assessed by FACS in cells incubated with SWCNTs (0.025 mg/mL) for 6, 12, 24 and 48 h. **a** Representative FACS analyses shown in the histogram format. **b** *Bar graph* showing that SKHep 1 cell viability is not altered by CNT exposure at different time points. Reproduced from Ladeira et al. with permission from Nanotechnology [20]

2.3 The Use of SWCNTs for siRNA Delivery In Vivo

The potential of different functionalized SWCNTs to act as efficient siRNA delivery agents in vivo has been demonstrated in several studies and most of these studies are focused on the use of SWCNTs as siRNA platform to suppress tumor growth. Yang et al., [15] demonstrated that intravenous injection of the complex SOCS1siRNA:positively charged SWCNTs (SWCNTs +) reduced SOCS1 (suppressor of cytokine signaling type 1) expression and retarded the growth of established B16 tumor in mice, suggesting that SWCNTs-based siRNA transfer system could be used in vivo for gene therapy. Following a similar strategy, Zhang et al. [16] targeted telomerase reverse transcriptase (TERT) and efficiently suppressed tumor growth and induced the senescence of tumor cells in animal studies corroborating the idea that functionalized SWCNTs enter cancer cells providing potent RNAi in vivo.

By using a very simple approach of noncovalently complexing siRNA to pristine SWCNTs Bartholomeusz et al. [24] achieved an efficient delivery of siRNA into cancer cells. In this work, the complex SWCNT:siRNA targeted to hypoxia-inducible factor 1 alpha (HIF-1α) was added to cancer cells and also administered in vivo to mice bearing MiaPaCa-2/HRE tumor. In both cases, it was observed a specific inhibition of HIF-1α activity, demonstrating the potential for using siRNA:SWCNTs complex in vivo as a therapeutic approach for cancer.

For the use of SWCNTs in biomedical application, the study of pharmacokinetics of CNTs is very important. Cherukuri et al. [25] performed an evaluation of pharmacokinetics of SWCNTs in rabbits intravenously administered with pristine SWCNTs dispersed in a solution of surfactant Pluronic. Blood sera were analyzed by near-infrared (IR) fluorescence spectroscopy and SWCNT blood elimination kinetics was measured. The authors observed that the nanotube concentration in the blood serum decreased exponentially with a half-life of 1.0 ± 0.1 h. Although this short half-life is not ideal for biomedical applications, there are many other parameters that regulate and improve the pharmacokinetics of CNTs, and among these we can cite the surface chemistry [26]. The pharmacokinetics of SWCNTs can be significantly altered by the type of suspending agent and functionalization method, with PEGylation being the most efficient method in improving the pharmacokinetics profile of CNTs. For instance, PEGylated-SWCNTs exhibit relatively long blood circulation times and high tumour accumulation [27].

By performing in vivo studies Wang et al. [28] have shown that SWCNTs can effectively delivery siRNA into tumor cells. The authors used polyethylenimine (PEI)-functionalized SWCNTs bound by DSPE-PEG2000-Maleimide conjugation with the asparagine-glycine-arginine (NGR) peptide. The NGR peptide is widely used to target tumor environments. Then, the resulting complex was used to delivery hTERT siRNA into PC-3 cells. The results showed that SWCNTs-PEI:siRNA-NGR complex induced severe apoptosis and suppressed proliferation of PC-3 cells. In animal studies, this complex exhibited higher antitumor activity, that was enhanced by the use of near-infrared (NIR) photothermal therapy. The combination of

nanomaterials with thermal therapy may represent a promising strategy in the field of cancer treatment. Huang et al. [29] also used PEI functionalized-CNTs for the delivery of siRNAs directed against glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Corroborating previous studies, PEI-NH-SWCNTs successfully delivered GAPDH siRNA into HeLa-S3 cells resulting in suppression of GAPDH mRNA. By using this method the authors achieved silencing efficiency comparable to a commonly commercial reagent.

The in vivo delivery of siRNA to therapeutically reduce cholesterol levels was accomplished by McCarroll et al. [30] who employed as nanocarriers SWCNTs functionalized with lipids and natural aminoacids-based dendrimers. Functionalized SWCNTs were conjugated with siRNA targeting apoliprotein B (ApoB), a protein involved in cholesterol metabolism, and the efficiency of this strategy was evaluated. The complex ApoB siRNA:SWCNTs was injected in mice via tail vein and led to approximately 50 % silencing of ApoB in liver followed by a decrease in ApoB plasma levels. Importantly, neither toxic effects nor activation of the immune system was observed in mice treated with the complex.

In conclusion, the last two decades have provided remarkable work in the field of nanomedicine and the biomedical applications of SWCNTs. Thus, these data highlight the fact that functionalized SWCNTs represent effective platforms for systemic in vivo siRNA delivery.

2.4 The Application of CNTs to Hard-to-Transfect-Cells

Cardiomyocytes and other primary cells [31] are usually considered hard to transfect cells, and unfortunately, efficient gene transfer in these cells is still limited. A very useful property of CNTs is their capability to penetrate hard-to-transfect cells, such as cardiac cells, as first demonstrated by Krajcik et al. [32]. In this work, the authors used SWCNTs functionalized with hexamethylenediamine (HMDA) and poly(diallyldimethylammonium)chloride (PDDA). These functionalized $_{f}$SWCNTs were then complexed non-covalently with negatively charged siRNA directed to ERK (extracellular signal regulated kinase) and used in a primary culture of cardiomyocytes. ERK is a serine/threonine kinase that is a member of the extracellular signal-regulated kinase family of proteins, which is activated in response to numerous stimuli such as growth factors, hormones among others and is involved in the regulation of multiple signaling pathways in cardiac cells [33]. As shown by the authors, the siRNA:PDDA-HMDA-SWCNTs construct entered cardiac cells and resulted in efficient ERK knockdown. This study showed for the first time the potential of SWCNTs to carry siRNA into cardiac cells. The use of SWCNTs as a tool for siRNA delivery in knocking down experiments in muscle cells was also assessed by Lanner et al., who showed potent RNAi of TRPC3 channels in adult skeletal muscle fibers exposed to TRPC3 siRNA:CNT complex [34].

Our group also demonstrated the ability of SWCNTs to efficiently enter and carry siRNA to primary culture of cardiomyocytes [20]. In this work, short single-wall carboxylic-functionalized CNTs were incubated with cardiomyocytes for 48 h and then examined by Raman spectroscopy. Figure 2.4 shows the presence of CNTs inside neonatal cardiac cells, further confirming the ability of these nanostructures to enter these cells.

The ability of carboxylated SWCNTs complexed to siRNA targeted to InsP3R-II to efficiently knockdown InsP3R was assessed by immunofluorescence experiments (Fig. 2.5). Primary culture of cardiac cells was exposed to the InsP3R-II-siRNA: CNT complex (CNT 0.0250 mg/mL and 100 nM of siRNA) and evaluated by immunofluorescence using specific anti-InsP3R-II antibody. In muscle cardiac cells, InsP3R-II channel is found in the cytosol and nuclear envelope, as shown in Fig. 2.5. In cells treated with the InsP3RII-siRNA:CNT complex, InsP3R-II labeling was significantly reduced when compared to control cells, confirming the fact that CNTs can act as efficient nanocarriers for siRNA to cardiac cells. We also tested whether InsP3R-II-siRNA:CNT complex would affect the expression levels of another intracellular Ca^{2+} channel in the cell, the Ryanodine Receptor (RyR).

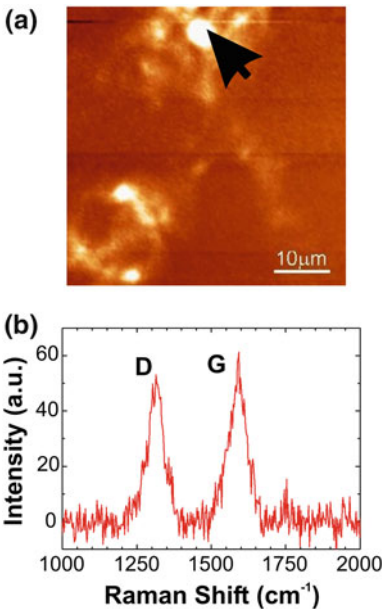

Fig. 2.4 CNTs can be uptaken by neonatal cardiomyocytes. The G band Raman peak at 1590 cm^{-1} was used for CNT detection inside the cell. **a** The plot of the carbon nanotube G band Raman intensity (see arrow) gives the CNT concentration inside the neonatal cardiomyocyte. **b** Representative Raman spectrum of neonatal cardiomyocytes exposed to CNTs (0.025 mg/mL) for 48 h taken from the area marked by the *black arrow*. The G and D carbon nanotube Raman peaks are highlighted. Reproduced from Ladeira et al. with permission from Nanotechnology [20]

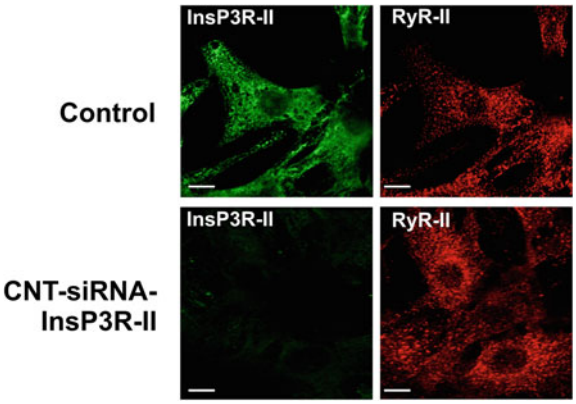

Fig. 2.5 Efficient reduction of InsP3R-II expression in neonatal cardiomyocytes exposed to InsP3R-II-siRNA:CNT complex. Representative immunofluorescence of neonatal cardiomyocytes double-labeled with anti-InsP3R-II (*left panels*), and anti-RyR-II antibodies (*right panels*). Neonatal cardiomyocytes were incubated with InsP3R-II siRNA:CNT complex (*bottom*) or with a suspension of CNTs *(top)* for 48 h, and double-labeled with anti-InsP3R-II and anti-RyR-II antibodies. SWCNTs efficiently delivered InsP3R-II siRNA (100 nM) into cardiac cells and reduced InsP3R-II expression in these cells. RyR-II immunolocalization was not altered in cells exposed to InsP3R-II siRNA:CNT complex. *Scale bar* 10 μm. Reproduced from Ladeira et al. with permission from Nanotechnology [20]

As shown in Fig. 2.5, RyR II staining and distribution throught the cells was not altered in cardiomyocytes exposed to InsP3R-II siRNA:CNT complex.

In gene knocking down experiments, CNT toxicity is a prime concern. In order to assess whether CNTs could alter cardiomyocyte viability in vitro we used a fluorescence-based live/dead assay that indicates the proportion of live and dead cells. In this experiment, cardiac cells were exposed to a solution containing CNTs for 48 h (final concentration 0.050 mg/mL). Figure 2.6 shows that incubation of neonatal cardiomyocytes with CNTs in a concentration superior to that used for siRNA experiments (0.050 mg/mL) does not alter the proportion of live/dead cells when compared with untreated cells. These findings indicate that carboxylated CNTs do not confer apparent toxicity to cardiomyocytes even when a concentration of CNTs superior to that used for the gene knocking down experiments was tested. Similar findings were obtained in a study performed by Garibaldi et al. [35] who demonstrated the biocompatibility of SWCNTs with cardiac muscle cells from a rat heart cell line H9c2. Experiments performed by Krajcik et al. [32] also confirmed the fact that functionalized cationic SWCNTs present no cytotoxic effects to cardiac cells. Taken together findings support the idea that functionalized SWCNTs confers low or no toxicity to cells in vitro [13, 22, 36–38].

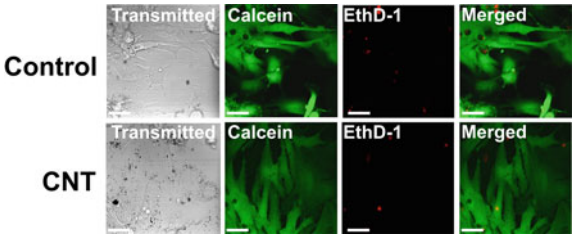

Fig. 2.6 CNTs do not alter cardiomyocyte viability. Cardiomyocytes were submitted to fluorescence-based live/dead viability assay. Dead cells were labeled with ethidium homodimer-1 (EthD-1) and living cells were labeled with calcein AM dye. Images of neonatal cardiomyocytes exposed or not (untreated control) to CNTs (0.05 mg/mL) for 48 h were collected in a confocal microscope. Reproduced from Ladeira et al. with permission from Nanotechnology [20]

2.5 Summary and Perspectives

In conclusion, based on available data in the literature, it is well established the fact that SWCNTs represent a very efficient system for siRNA delivery in a variety of cells, including cells that are hard to transfect, such as cardiomyocytes. In vivo the use of this nano-platform for siRNA based therapeutics offers great potential due to its ability to protect the siRNA from enzymatic degradation, efficient membrane penetration, and apparent low toxicity. However, regardless of the knowledge gained in recent years in biomedical applications of SWCNTs as nanovectors for siRNA, the number of studies is still limited, indicating that systemically targeting CNTs for therapeutic use remains challenging. Further work is warranted in order to optimize the siRNA:CNT formulation, the siRNA release and to achieve proper targeting of the complex. New studies should be considered in order to improve the pharmacokinetic profile of siRNA:CNT complex. In addition special attention should be taken in order to develop more efficacious route of delivery in vivo while maintaining controlled gene expression.

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Chapter 3
Carbon Nanotubes as a DNA Delivery Agent for Generation of Genetically Modified Mammals Embryos

Humberto de Mello Brandão, Michele Munk, Bruno Campos de Carvalho, Alessandro de Sá Guimarães, Wanessa Araújo Carvalho, Juliana Carine Gern and Luiz Sergio Almeida Camargo

Abstract Several research centers and pharmaceutical corporations routinely use genetically modified animals (GMAs) in the development of new drugs, in the identification of new drug targets and to test drugs' efficacy and safety. The most usual methods to produce GMAs are pronuclear microinjection, somatic cell nuclear transfer, retroviral vectors, and recently, embryonic stem cell transgenesis. These methods make use of DNA vectors and present several limitations. Recently, nanomaterials have been applied as an alternative vector for delivery of exogenous DNA into mammalian cells. This chapter addresses the use of carbon nanotubes (CNTs) as a DNA delivery agent for the generation of genetically modified mammals embryos. CNTs can be easily bound to DNA by non-covalent attachement. The DNA strand spontaneously wraps around the carbon nanotubes and DNA molecules can be encapsulated within or around them. The process of

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interaction of DNA/RNA with CNT favors their protection from degradation by cytoplasmic nucleases, increasing the integration of the transgene into cell nucleus. Thus, the use of CNTs can be far simpler and less laborious when compared to other techniques to produce GMAs.

3.1 Introduction

Genetic engineering aims to intentionally remove, modify or add genes to DNA molecule in order to improve the development of new therapies and new disease models; to study the mammalian gene function; and to generate genetically modified organisms (GMO) and genetically modified animals (GMA) [1, 2].

Several kinds of cells have undergone gene manipulation procedures, such as mesenchymal cells, embryonic stem cells, embryos and others [1–4]. The most usual methods to produce GMAs are pronuclear microinjection, somatic cell nuclear transfer, retroviral vectors, and recently, embryonic stem cell transgenesis [1, 3, 5]. All these methods make use of DNA vectors and require micromanipulation of the embryo or oocyte as a limitation. In addition to said limitation, others can arise depending on the DNA vector used, such as restricted targeting of specific cell types, toxicity, low efficiency, limited DNA carrying capacity, production problems, recombination occurrence, and high cost [3, 6, 7].

Nanomaterials have been applied as an alternative vector for delivery of exogenous DNA into mammalian cells. The size reduction of the DNA delivery vehicles is crucial for the diffusion of the complex DNA-vector in biological systems [8]. Several nanomaterials, such as chitosan nanoparticles [9], gold nanoparticles [10] and halloysite clay nanotubes [5] have been used for DNA delivery. However, carbon nanotubes (CNTs) have gained more attention due to their greater biocompatibility, capacity to penetrate cell membranes and distribute themselves into the cytoplasm and the nucleus of cells [4], greater DNA protection [11, 12], and simple DNA immobilization [13]. Among all these nanomaterials, only halloysite clay nanotubes have been used to produce genetically modified mammal embryos. However, by means of a method which is poorly reproducible.

In this chapter we will discuss the use of carbon nanotubes (CNTs) to generate transgenic animals. Specifically, we will review various approaches to making mammals embryos transgenic, discuss their applications, and consider their relative advantages and disadvantages. We review the interaction of CNT and DNA and finally to provide an approach for toxicological studies that may precede CNT use.

3.1.1 Oocyte and Embryo Structures

The ovary has two key functions, the production of gametes and the production of hormones. The ovarian follicle is the structure which enables both functions. During fetal life and/or shortly after birth (depending on species), a large number of

primordial follicles are formed, which will act as a follicle source which will help to supply the post-pubescent individual's estrous cycles. Initially, the follicle is composed of an oocyte surrounded by a unicellular layer of granulosa cells. During the follicular phase, the granulosa cells multiply and differentiate into some theca cells. Concurrently, the oocyte begins its growth, which is virtually complete with the formation of the follicular antrum. During this period, in its initial phase, the deposition of the Pellucid Zone (*Zona Pellucida*—PZ) occurs. During the follicular growth, the oocyte meiosis remains at rest (*dictyate nucleus*), being normally re-established with the extrusion of the first polar corpuscle (the moment of occurrence varies depending on the species) and their ovulation, when the oocyte maturation continues [14–16].

The oocyte maturation and the meiosis are only concluded with the fertilization process, when the oocyte becomes a zygote. In order for the sperm fertilization to occur, it must firstly be able to transpose the ZP (aided by enzymes present in its acrosome) and merge with the plasma membrane of the oocyte. After such a fusion, the male genetic material is introduced into the cytoplasm of the oocyte, which expels the second polar corpuscle. The maternal chromosomes (haploid) are then coated with a pro nucleus, while the head of the sperm forms the male pronucleus. Both male and female pronuclei are directed to the center of the zygote where they merge, initiating the process of parental gene transcription (paternal and maternal) and cleavage. After the sixteen-cell stage, the embryo is called a morula (Fig. 3.1a),

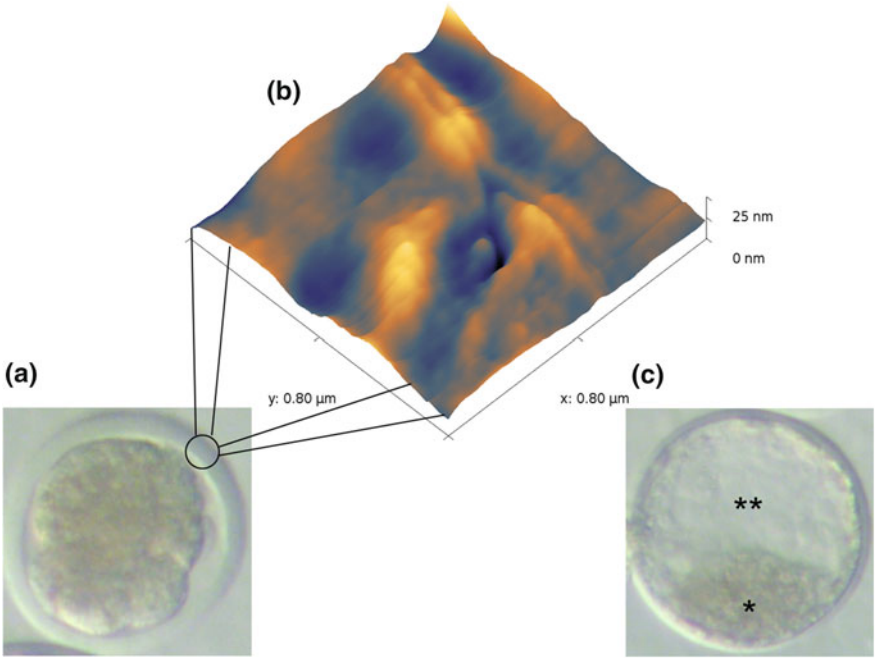

Fig. 3.1 **a** Micrography of bovine morula. **b** Atomic Force image of bovine Zona Pellucida (supported by Bernardes-Filho R). **c** Micrography of bovine blastocyst. * Embryoblast and ** blastocoele

and when the blastocoele begins to take form it is given the name of blastocyst. A blastocyst (Fig. 3.1b) is composed by the embryoblast (which will originate the fetus) and by the trophoblast (wich will originate the fetal membranes).

The ZP (Fig. 3.1c) is the biggest obstacle in the production scheduling of genetically modified embryos, it follows the whole embryonic evolution until its outbreak, and it is present in all mammalian oocytes. The ZP is formed by an acellular secretion of sulphated glycoproteins, mostly composed (95 %) by monomers ZP1 (~200 kDa), ZP2 (~120 kDa) and ZP3 (~83 kDa) [17–19]. ZP2 and ZP3 form dimers of a large filamentary polymer. This polymer is non-covalently crosslinked by ZP1. It is estimated that there are connection points every 75–85 nm in the polymer [19]. This forms a hydrogel which coats the embryo/oocyte and can account for up to 10 % of its diameter [18, 19]. Immediately after the oocyte fertilization, a phenomenon known as cortical reaction occurs, which is characterized by intense exocytosis of granules located in the periphery of the oocyte. Probably, these granules contain peroxidase and protease which convert ZP2 to ZP2f, resulting in increased polymer interchain crosslinking [19]. The final result is a spongy structure, rich in small pores, which allows the passage of nutrients into the internal structure of the ZP and, simultaneously, prevents polyspermy as well as the penetration of particulate structures such as viruses.

3.1.2 Applications of Genetically Modified Animals (GMA)

The first transgenic mice were made by the retrovirus-mediated techniques of Rudolf Jaenisch, a biology professor at Massachusetts Institute of Technology [20]. In the early 70s, Jaenisch showed that by injecting the Simian Virus 40 into mice in their early embryonic period, virus incorporation into the embryo genome would be induced, thus transmitting the virus to the embryo offspring. Using the same concept with the Moloney leukemia virus, Jaenisch induced leukemia in successive generations of a genetically modified mouse [21]. Jaenisch studies were the basis for the current production of laboratory GMA, which can have its genome changed to promote gene insertion, conditional expression, gene over/down expression, or gene knockout. This enabled the development of new animal models to understand mammalian physiology and physiopathology, humans' susceptibility to diseases, disease progression and its response to pharmaceutical treatments, as seen in the Table 3.1. Several research centers and pharmaceutical corporations routinely use GMAs in the development of new drugs, in the identification of new drug targets and to test drugs' efficacy and safety [22–24].

Another approach made in the pharmaceutical segment is the use of GMA as bioreactors to express heterologous proteins (proteins from other species). The animals used for such a purpose are livestock, e.g. cows, goats, sheep and pigs. The GMAs, when compared with genetically modified bacteria, can synthesize recombinant proteins with the appropriate post-translational modifications [7]. Consequently, the protein from GMA may be less immunogenic and present better

Table 3.1 Products made from genetically modified animal

Animal	Product	Potential use	Technique	References
Mouse	Septin- SEPT4	For Parkinson's disease study	Pronuclear microinjection	[39]
Bovine	n-3 fatty acid desaturase	Ômega-3 fatty acids production	Somatic cell nuclear transfer	[40]
Pig	Complement regulatory proteins CD55 (hDAF), CD59 and α-1,2 fucosyltransferase	Xenotransplant	Pronuclear microinjection	[41]
Pig	Human membrane cofactor protein	Xenotransplant	Pronuclear microinjection	[42]
Dairy cattle	Recombinant human lactoferrin	Human lactoferrin production	Pronuclear microinjection	[43]
Rabbit	Recombinant human factor VIII	Human factor VIII production	Microinjection	[44]
Goat	Human coagulation factor IX	Human coagulation factor IX	Somatic cell nuclear transfer	[45]
Mouse	Thioredoxin-1	For diabetic osteopenia study	Pronuclear microinjection	[46]
Mouse	Cardiac-specific IGF-1 Receptor	For diabetic cardiomyopathy study	Pronuclear microinjection	[47]
Goat	Human Lysozyme	For protection against infection	Pronuclear microinjection	[48]
Goat	Human Granulocyte Stimulating Factor	Production of valuable protein of pharmaceutical interesting	Pronuclear microinjection	[49]
Rat	P53 gene knockout rat	Creation of a model for human diseases	Embryonic-stem cell trangenesis	[50]
Rat	Knockout rat	Creation of a model for human diseases study	Zinc-finger nucleases	[51]
Rat	Knockout rat	Creation of a model for human diseases study	TALENs	[52]
Marmoset	Green fluorescent protein (EGFP)	Creation of a non-human primate model for human diseases study	Retroviral vectors	[53]
Sheep	Knockdown of myostatin expression	Enhance of muscle growth	Somatic cell nuclear transfer	[54]
Mouse and Goat	Recombinant human butyrylcholinesterase	Protect against organophosphate poisoning	Pronuclear microinjection	[55]

bioactivity than the protein produced by transgenic bacteria [7]. On the other hand, heterologous proteins produced in GMAs can be selectively expressed in a specific tissue, and then collected and purified from its secretions. Heterologous proteins are extracted from blood, urine, seminal plasma and milk [7, 25]. However, the expression of proteins in milk is apparently more promising, because the production of heterologous protein is efficient and the extraction process is easier. According to Houdebine [25], the global demand of human serum albumin, alpha-1-antitrypsin, anti-thrombin-III and blood coagulation factor IX can be supplied by milk production from 5400 transgenic cows, 4300 transgenic ewes, 43 transgenic goats and 4 transgenic pigs, respectively. To explore the market for heterologous proteins, several start-ups and affiliates of large publicly traded companies were created in the past decade. Only in North America and Europe, at least 11 biotechnology companies were registered in 2012 [3]. Recently, Biosidus S.A., an Argentinian biotechnology company, announced the production of recombinant human insulin and human growth hormone in transgenic cow milk [26, 27]. Currently, there are at least 10 heterologous proteins produced in milk under clinical trial [25], and two of them have been approved for human use by the European Medicines Evaluation Agency and the United States Food and Drug Administration [3].

The prospect that the xenotransplants could provide a solution for the global deficit of transplanting organs and tissues boosted the biomedical researches in this area. The Australian National Heart and Medical Research Council define xenotransplantation as “the term used to cover the transplantation of living cells, tissues or organs from one species to another” [28, 29]. The biggest challenge to xenotransplant success is the immunological barriers between species. Using transgenic animals with gene insertion or gene knockout is the most promising approach to “fool” rejection mechanisms and incompatibilities between the transplanted organ donor and the recipient [29]. However, there are questions regarding xenotransplants related to the risk of interspecies viral transmission, ethical issues, as well as their efficacy [28].

The GMAs can also be used to improve food quality and production. One of the biggest challenges for the next three decades will be feeding a global population with greater purchasing power (in developing countries) and thirty percent higher than the current seven billion people [30, 31]. In this context, it is estimated that the current global food production should be increased over seventy percent until 2050 [32, 33], but global climate change can lead to a decrease in the food production and there is little new land which can be brought into production on the Earth [33, 34]. Similarly, since grain production is increasing by the use of approved transgenic crops, GMAs may contribute to increase global food production. The GMAs have potential to improve food animal production via improved meat and milk production; enhanced animal fertility; increased resistance to disease; enhanced animal adaptation to adverse climate, and production of animals with reduced environmental impact [35, 36]. After international harmonization, all the potential of GMAs can be put into practice due to the existence of a consolidated market of livestock embryos. According to the International Embryo Society, the embryo transfer industry is growing, promising and healthy on a worldwide scale and 1.25million of livestock embryos were produced worldwide in 2011.

Nutraceuticals are foods "which provide medical or health benefits, including the prevention and/or treatment of a disease" [36]. Insertion, upregulation or knockout of genes which encode the production of bioactive molecules with beneficial or dangerous effects, can be used to enhance the quality of food produced by livestock. Thus, animal body composition and milk composition can have their protein and fat acid increased or changed. The company Biosidus [27] intends to launch a transgenic cow milk in the Argentinian market, which contains antibodies against rotavirus. This virus is a major cause of child death in emerging and poor countries.

Table 3.1 shows some examples of products made from GMAs which were generated by transgenic embryos. More examples can be seen in reviews [1–3, 7, 25, 37, 38].

3.2 Conventional Gene Transfer Methods for Generating Transgenic Animals

3.2.1 Pronuclear Microinjection

The most commonly used method to generate transgenic animals, mainly for mice, consists of injecting genes into the pronuclei of embryos, method which was reported in 1980 [56]. Such a technique consists of immobilization of the one cell embryo, identification of the male pronuclei, microinjection of DNA directly into the male pronuclei and visualization of nuclear volume increase (Fig. 3.2a) [57].

In mice, the efficiency oscillates around an average value of 30 % [58]. Thus, such methods allowed the rapid advancement of transgenic biotechnology in this species, so that there are currently numerous commercially available transgenic strains of mice for studies regarding human diseases. However, as for domestic animals, such as sheeps, cows and goats, the pronuclear microinjection does not present the same results as it does in mice for a number of reasons. In general, ruminant zygotes are too dark in color compared to those of mice. This feature makes the pronuclei visualization difficult for the correct microinjection of the DNA of interest. Thus, the pronuclei are not visible or accessible [59], making the generation of a transgenic animal a laborious task. Another major problem in this method is that the integration of the foreign gene in domestic animals is either extremely low [7] or leads to random integration of the foreign gene, producing chimeric animals. The chimerism reduces the transmission rate of the altered genes of the progeny to their offspring, since there is a large number of transgenic germ cells. Thus, it is only possible to have a non-chimeric animal after the birth of the second generation. In species with long generation intervals, such as bovine (4–5 years), this waiting can be costly. In general, the pronuclear injection efficiency in ruminants and other domestic species does not exceed 10 % [56, 60, 61], making it less attractive for ruminants.

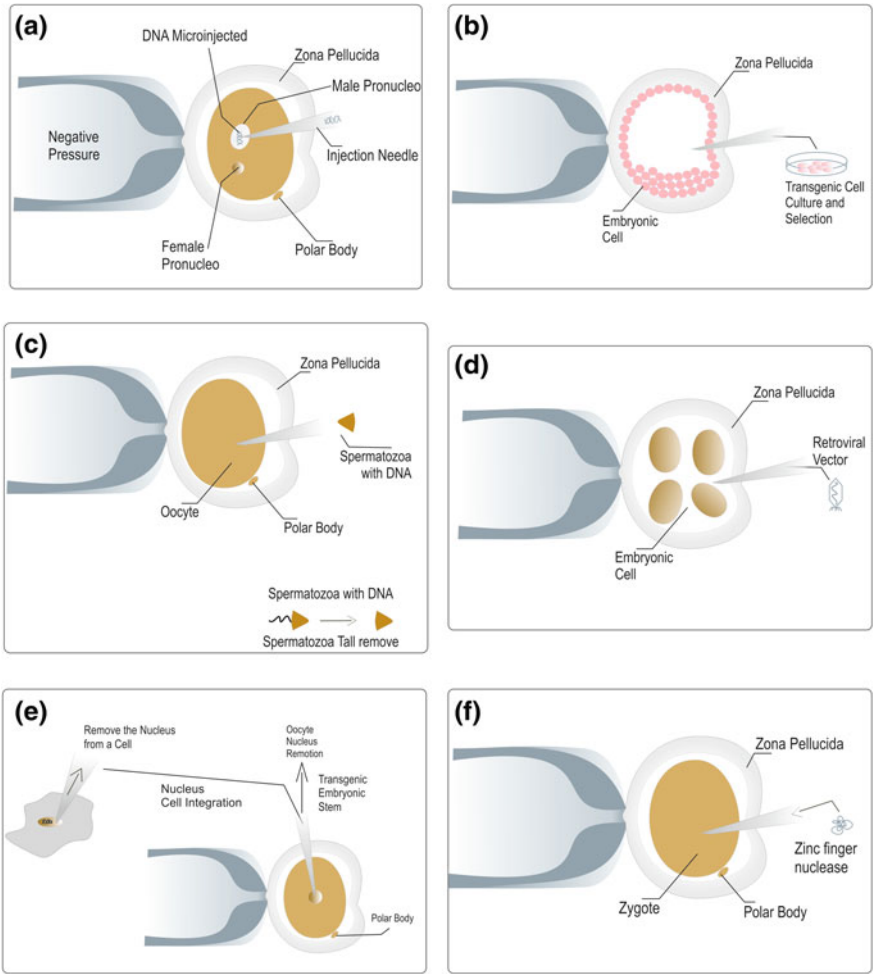

Fig. 3.2 **a** Schematic representation of the pronuclear microinjection technique at genetically modified animals production. **b** Schematic representation of the embryonic stem cell transgenesis technique at genetically modified animals production. **c** Schematic representation of the Sperm-mediated gene transfer technique at genetically modified animals production. **d** Schematic representation of the retroviral vector technique at genetically modified animals production. **e** Schematic representation of the somatic cell nuclear transfer technique at genetically modified animals production. **f** Schematic representation of the Zinc-Finger nuclease technique at genetically modified animals production

3.2.2 Embryonic Stem Cell Transgenesis

Embryonic stem (ES) cells have afforded a means of directly modifying the animal genome [62–64]. The advantages of this method consist of two hallmark properties: self-renewal and ability to remain in their undifferentiated original state. With this,

genetic modification and selection of cells during in vitro culture is possible, followed by transfer to the blastocyst, which usually generates chimera animals. For this reason, the ES lines are isolated from the inner cell mass of a blastocyst and then cultured, so that their undifferentiated status is maintained. In order to genetically create modified embryos from the ES cells, it is necessary to modify the DNA of the ES cell, to select transgenic ES cells from a culture medium and then microinject the transgenic ES cells into the blastocyst or morula (Fig. 3.2b).

Such a technique has been used for *gene targeting*, allowing the transgene integration DNA site-specific and the generation of animals by gene mutations, such as substitutions, deletions and inversions. However, in species other than mice and humans, the establishment of true stem cells has not been recorded [65]. Furthermore, the chimera would still be a problem for species with long generation intervals.

3.2.3 Sperm-Mediated Gene Transfer (SMGT)

Genetic materials are introduced into the sperm, which is used to fertilize eggs. In 1989, Lavitrano et al. [66] showed that mouse sperm has the capacity to capture foreign DNA and to transfer it into the egg at fertilization. The transgenic spermatozoa can be used in natural fecundation, in vitro fecundation or directly microinjected in the oocyte to produce GMAs (Fig. 3.2c). In vitro transgenic spermatozoa can be obtained by electroporation, by restriction in the enzyme-mediated integration or by intracytoplasmic sperm injection after sperm/DNA interactions [67]. On the other hand, in vivo transgenic spermatozoa can be obtained by microinjection of exogenous DNA, either directly into the seminiferous tubules or into the rete testis; or by transplantation of genetically transformed germ cells to the testicle [67]. Thereafter, genetically modified animals of other species are generated [68, 69], but the technique is still controversial because the results are not always reproducible [58, 70, 71].

3.2.4 Retroviral Vectors

Retroviral vectors are a tool commonly used to deliver genetic material into cells. Chan et al. [72] used retroviral vectors to insert a transgene into a zygote, injecting it into the perivitelline space (Fig. 3.2d). However, one of the problems with this technique is that it represses the transgene expression [73]. New vectors were developed from the HIV-1 virus to solve this problem, by allowing the birth of genetically modified mice, pigs and cattle [74, 75]. Those vectors, called lentiviral, have no genes associated with viral infectivity and have been improved to avoid self-activation, viral recombination and activation of oncogenes [76]. Lentiviruses have been used to produce genetically modified domestic animals [77]. However, their usage may be limited by public acceptance.

3.2.5 Somatic Cell Nuclear Transfer (SCNT)

One of the methods which provided a great stimulus for gene modification in domestic species is somatic cell nuclear transfer (SCNT). This process was used to create Dolly [78] by transfer of a donor nucleus into the cytoplasm of an enucleated MII oocyte (Fig. 3.2e). A modification of this process is performed by using unfertilized oocytes whose membranes are fused with membranes of an adult transgenic cell. Schnieke et al. [79] first reported the usage of this technique to produce a sheep to secrete human coagulation factor IX. This technique has been preferred for many experimental generations of genetically modified animals [80, 81], but such methods lead to a higher number of fetal and perinatal losses, generating few viable animals [82], regardless the existence of genetic modification, with losses of up to 99 %. The most intensive use of SCNT for transgenesis will depend on the understanding of the causes of such losses as well as the improvement of the reconstruction process of an embryo by using somatic cells.

3.2.6 Zinc-Finger Nucleases (ZFN) and Transcription Activator-like Effector Nucleases (TALENs)

Among the new techniques which have appeared are the nucleases, such as Zinc-Finger nucleases (ZFN) and transcription activator-like effector nucleases (TALENs). These molecules are able to cleave the double-stranded DNA molecules which are not repaired by joining the cut homologous ends, leading to short deletions in the gene sequences in the cleavage site and thereby inactivating the gene.

The enzyme FokI, a restriction endonuclease, is associated with proteins that have a specific DNA cleavage domain [83], creating hybrid molecules, which can be used to cleave a specific sequence in the genome, thus causing gene silencing or allowing the introduction of a gene modification by homologous recombination [52]. ZFN and TALENs have been the hybrid molecules used to genetically modify the genome of mice and rats [51, 52, 84] and, more recently, in pigs and cattle [85]. The simple cytoplasmic injection of TALENs in oocytes and zygotes was enough to cause directed mutations in the genome of the embryo (Fig. 3.2f). These hybrid molecules open up a new perspective for the modification of the genome of the domestic species.

3.3 Carbon Nanotubes (CNT) as a Gene Transfer Agent

3.3.1 Interaction of CNT and DNA

Carbon nanotube (CNT) can be easily bound to DNA by non-covalent attachment [86]. The DNA strand spontaneously wraps around the CNT [87, 88]. Gao and Kong [89], using molecular modeling, found that DNA molecules can be

encapsulated within or around the CNT. The nucleotide bases interact with the CNT by hydrophobic interactions. This occurs via π-stacking taking place between the aromatic rings of the DNA bases and the CNT, while the phosphate groups of the DNA molecule interact with water [90]. CNT probably binds itself to the duplex DNA major groove [91], since this association may be an enthalpy-driven process. However, the complex DNA/CNT formation may be variable, since each nucleobase is oriented in distinct ways, related to the CNT long axis [92]. Another type of interaction between DNA and CNT can occur via attachment of 5′ or 3′ ends of a DNA molecule onto the surface of the CNT, because of the partially exposed hydrophobic base pairs at the ends [93].

Many parameters influence transfection efficiency using CNT, including the various methods of CNT functionalization [94]. The CNT functionalization can be noncovalent or covalent [4]. In noncovalent functionalization, the surface of the CNT is coated with several types of molecules which interact with the CNT via π-π stacking or by means of hydrophobic interactions [86]. For this purpose, the functionalization is performed with surfactants, polymers, proteins and other amphiphilic molecules [4, 86]. In covalent functionalization, the intrinsic sp^2 structure of the CNT sidewall, i.e. the original electronic structure is changed and, consequently, its properties are changed [4]. Covalent functionalization can be carried by several routes, e.g. oxygenated functional groups (carboxylic acid, ketone, alcohol and ester groups), amidation, reduction of nitro groups, cleavable disulfides, fluorination, electrophilic addition, cycloaddition and others [4, 86].

Regardless the type of CNT functionalization (covalent or noncovalent), in general, positively-charged CNT has better association with DNA than uncharged CNT or negatively-charged CNT, since DNA is negatively charged. For example, Pantarotto et al. [95] reported that NH_3-funcionalized CNT were more strongly associated with plasmid DNA by electrostatic interactions. Multi walled carbon nanotube- polyamidoamine (MWCNT-PAMAM) hybrid possessed a good recombinant plasmid enhanced green fluorescent protein-N1 (pEGFP-N1) immobilization ability and could efficiently deliver green fluorescent protein (GFP) gene into cultured HeLa cells [96].

When the CNT is in suspension, the physicochemical parameters of the liquid phase must also be taken into account in the association between the DNA and the CNT. Wan and coworkers [97] reported that the pH drove a reversible assembly of DNA-wrapped single wall carbon nanotubes (SWCNTs). Ladeira and coworkers [98] used ultrasound to coil siRNA into carboxyl-functionalized single-wall carbon nanotubes (see Chap. 5 on this book). The use of ultrasound during DNA/CNT association can be favorable to expose the DNA binding domains with the CNT, probably due to the formation of a cavitation area and consequent increase in the local temperature. Thus, this procedure improves the association between DNA and the CNT. By using a similar methodology, we have shown that a large segment of DNA can be associated with a MWCNT-COOH after ultrasonication [99]. The complex pGFP: MWCNT-COOH was able to transfect bovine fibroblast cells (Fig. 3.3a). However,

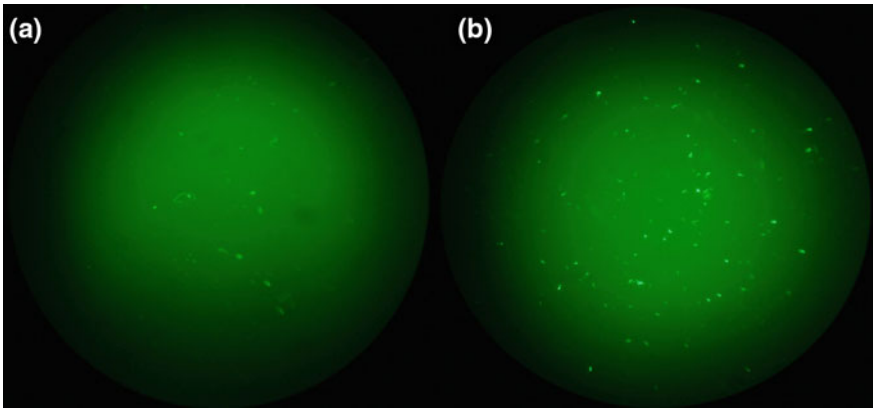

Fig. 3.3 The fluorescence of bovine fibroblast cells successfully transfected with GFP gene: **a** MWCNT-COOH with GFP gene; **b** poly(ethyleneimine) with GFP gene [99]

the amount of gene or protein conjugated by MWCNT-CHOO is still limited, resulting in low transfection efficiency (Fig. 3.3a) when compared to the reagent poly (ethyleneimine) (Fig. 3.3b).

3.3.2 Contribution of CNTs to Conventional Techniques

In somatic cells, embryonic-stem cells or in sperm-mediated gene transfer, several methods have been developed for the establishment of an efficient gene delivery system, such as viral vectors [100–102] or nonviral ones, like liposomes [5, 103], physical or chemical methods [104–106]. The transfection with viral vectors has shown good results due to their natural ability to penetrate cells and to take control over the DNA replication, RNA transcription and translation. However, these vectors have some limitations, such as the possibility of causing immune response against exogenous protein [107], high toxicity, low gene capacity, besides the difficulty of being used in large scale [108–112]. Multiple alternative approaches for non-viral gene delivery have been reported in the literature for in vitro transfection of mammalian cells, with limited success. The physical and chemical methods which operate in modifying the permeability of the cell membrane have their usage limited mostly due to toxicity [113, 114] and the plasmid DNA is generally degraded in the cytoplasm before reaching the nucleus [12, 115].

The process of interaction of DNA/RNA with CNT favors their protection from degradation by cytoplasmic nucleases [12], increasing the integration of the transgene into cell nucleus [115]. In addition, non-covalent binding of nucleic acids to the surface of the CNT increases the efficiency in releasing the contents into the cell [116, 117]. The CNT penetrate into cells by endocytosis or pass freely through the lipid bilayer in various types of somatic cells [94, 118], and mediate the

internalization of biomolecules such as iRNA and plasmid DNA into various mammalian cells [98, 119]. Thus, the use of CNT in the gene transfection process in different cells [12, 107, 115–118] has opened new possibilities for an efficient delivery in conventional methods for the generation of genetically modified mammal embryos (i.e. somatic cells, embryonic-stem cell or in sperm-mediated gene transfer).

3.3.3 *Potential DNA Delivery to Embryo Without Micromanipulation*

In embryos, one of the barriers found in the methods traditionally used for mammalian cell transfection is the presence of the ZP.

The ZP is a glycoprotein coat which plays an important role in the early stages of the embryonic development, performing functions such as blocking polyspermy, protecting cleaving embryos, as well as protecting them as they traverse the female reproductive tract [15]. This glycoprotein forms a "sieve-like" structure around the bovine embryo, preventing the free passage of nanospheres greater than 40 nm [120]. Therefore, usual transfection methods are not efficient for mammalian oocytes and embryos, since they are exclusively for somatic cells. Thus, the strategy of using membrane fusions for introducing vectors does not reach its goal of carrying the vector into the gamete or embryo, and transfection method should enable the DNA to penetrate the ZP. Additionally, all the techniques currently available for producing mammalian transgenic embryos are quite laborious, because each embryo must be individually micromanipulated. This manipulation may diminish the viability of the embryo.

Recently, to circumvent such limitations, our research group demonstrated that the CNT can cross the ZP [99]. Previously, we evaluated the citotoxicity of mutiwalled carbon nanotubes (MWCNT) on the mammal embryo development. Using bovine embryos produced in vitro and exposed to MWCNT, a slight increase in the apoptotic index was identified in the TUNEL test regarding the MWCNT exposed group. However, differences were not found ($P > 0.05$) in the hatching rate (natural ZP rupture) between the control and the treated groups. These results suggest that bovine embryos can resist to lower concentrations than 0.2 $\mu g.mL^{-1}$ of MWCNT in culture medium.

To prove whether MWCNTs can cross through the ZP, the embryos were washed several times with a medium without MWCNT, and then Raman spectroscopy was performed, coupled with confocal microscopy. Figure 3.4a shows small clusters of MWCNT on the inside of the embryo. Clusters of MWCNTs were probably formed due to embryo dehydration during the analysis. Figure 3.4b represents the Raman intensity of the G band (a characteristic spectral feature of the MWCNT appearing at 1580 cm^{-1}) along of microscopy Z axis. The G band intensity profile along Z confirmed the presence of MWCNT inside the embryo.

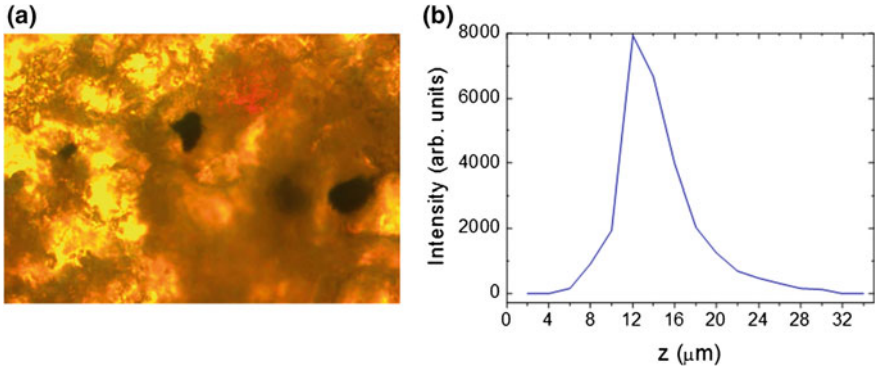

Fig. 3.4 **a** Confocal micrography of bovine morula with small clusters of mult-wall carbon nanotube. **b** Raman intensity of G band at microscopy Z axis, during the embryo scan [99]

The ability of the CNT to cross the ZP without impairing embryo viability makes the CNT a promising DNA vector to be used in the production of transgenic embryos. However, its embryotoxic effect, if any, need to be established. For this purpose, several further studies should be carried out, taking into account the CNT concentration, the exposure time and its effects, especially on genotoxicity.

3.4 Genotoxicity

3.4.1 General Aspects

Genotoxicity can be defined as the damage to genetic material by chemical agents, either endogenous or exogenous, which can cause DNA breakage or oxidation, thereby inducing the formation of cell micronuclei. Among the agents which have the potential to induce DNA damage, reactive oxygen species and reactive nitrogen species (ROS and RNS), aromatic compounds, as well as some kinds of particles and fibers, such as quartz and asbestos, play important roles. As for particles and fibers, in particular, genotoxicity could be classified as [121]:

- Primary genotoxicity: induced in the absence of an inflammatory response;
- Secondary genotoxicity: driven by the activation of inflammatory cells, such as neutrophils and macrophages, which are the main source of ROS and RNS.
- Direct genotoxicity: results from physical interactions of particulates with the genomic DNA;
- Indirect genotoxicity: results from increased production of ROS through the interaction of nanoparticules with other organelles such as mitochondria, for example. This can also result from the depletion of intracellular antioxidants induced by nanoparticules.

The accumulation of ROS, one of the main causative agents of genotoxicity in eukaryotic cells, is prevented by antioxidant molecules such as glutathione, thioredoxin and superoxide dismutase (SOD) [121]. Enzymes are also responsible for the direct restoration of damaged DNA strands to their original shape through repair and nucleotide excision. When these protective mechanisms fail, the DNA-damaged cells may undergo death by apoptosis or necrosis. If these mechanisms fail, the fixation of mutations occurs, culminating in the possible development of carcinogenesis.

Many kinds of mutations in genes are essential for the development and maintenance of physiologic processes. These mutations can be divided into proto-oncogenes, which are responsible for carcinogenic cell's growth and proliferation, tumor-suppressor genes, which inhibit its proliferation, or they are related to genes involved in the repair of DNA, such as the p53 gene [121–123]. DNA mutations also play an important role in the development of pathologies and in the modulation of susceptibility to disease. Mutations may involve either relatively short sequences, like the individual genes, or may occur in a larger scale. Point mutations can be divided into [121]:

– Transitions and transversions: replacement of one nucleotide with another;
– Inserts: addition of nucleotides;
– Deletions: removal of nucleotides.

Mutations can also affect chromosomal structures and are classified into:

– Amplifications: increased numbers of chromosome regions;
– Deletions: lower or deletion in the number of chromosome regions;
– Translocations: exchange of non-homologous chromosomes between regions;
– Interstitial Deletions: parts of a chromosome are removed, potentially resulting in the encoding of fused proteins;
– Chromosomal Inversions: rearrangement of segments within the same chromosome;
– Loss of heterozygosis: loss of one allele of a gene in regions in which the other allele has previously been inactivated.

When the chromosome segregation between daughter cells is disturbed during mitosis, there may be changes in the number of chromosomes, also called aneuploidy. Although much attention has been drawn to determine the possible genotoxic effects of CNTs, many questions are still unanswered, mainly due to the recent approach to emerging applications of these materials.

Genotoxicity is considered a useful tool for understanding the potentially carcinogenic risk of nanoparticles. In order to evaluate such an effect, several tests are used to identify broken DNA strands, determination of mutations, activity of molecules related to carcinogenesis, chromosomal aberrations and DNA oxidation [121–123].

3.4.2 Detection Techniques, Toxicity and Genotoxicity of CNTs

The use of CNTs for therapeutic and transgenic embryos must be very cautious, and several techniques are available for evaluating the genotoxicity of these nanoparticles. The use of CNTs for development of transgenic animals is not documented although there are few reports about its genotoxicity in zebrafish and avian embryos [124–127]. Once the size, the amount and the type of carbon nanoparticles (SWNTs or MWNTs) are crucial for determine its genotoxicity, the results provided in literature show discrepancies which should be investigated in order to optimize the use of CNT in the field of transgenesis.

So far, some genotoxicity assays demonstrated the effect of CNTs on different cell types and lineages (summarized at Table 3.2). All of these studies demonstrate a clear effect of amounts and particle size in genotoxicity. The cells exposed to CNTs could present one or more genotoxity effects at the same time, being the DNA breaks the most observed. CNTs genotoxicity are broadly studied in lung cells because of its importance for human health, especially for people that work with minerals and in metal industry, which are naturally exposed to nanoparticles that seems to be extremely toxic at higher levels (summarized at Table 3.2). The most widely used tests for determining genotoxicity are the Comet Assay, phosphorylation of histone H2AX, Fish, micronuclei formation and comparative genomic hybridization. This technics provides information about DNA breaks and oxidation, chromosomal aberrations and mutations.

The Comet Assay is a technique of microelectrophoresis made under neutral and alkaline conditions. This technique is reproducible, being able to simply and rapidly detect the presence of double strand DNA breakage (DSB), occurring under neutral conditions, and single strand DNA breakage (SSB), occurring in alkaline conditions. This technique makes it possible to assess DNA damage by using single-cell samples on agarose film-coated slides, which are submitted to an electrophoretic field and fluorescent staining. Samples presenting DNA damage form a “comet tail”, while samples with intact DNA migrate as a unique “point” in the electrophoretic field. DNA breakage induced by CNTs was described in mammalian species, especially human and mouse (summarized at Table 3.2). As already discussed it seems to be dependent of particle size and amounts of exposure which is determinant for cell death or development of mutagenesis which could culminate in carcinogenic effects.

The detection of phosphorylation of histone H2AX: H2AFX (H2A histone family, member X) is one of several genes encoding histone H2A. In humans and other eukaryotes, DNA is wound around groups of histones H2A, thus contributing to the formation of the DNA structure. The phosphorylation of H2AX at serine 139, also called gamma—H2AX, participates in the DSB DNA breakage reaction. This phosphorylation is triggered by the activation of intracellular kinases. The modification can occur accidentally during cell replication, either in response to genotoxic compounds and treatments, or during controlled physiological processes, such

Table 3.2 Genotoxic effect of carbon nanotubes on different cell types

Genotoxic effect	Cells	References
DNA breaks	Human mesothelial cells	[128]
	Mouse embryo fibroblasts	[129]
	Bronchial epithelial cells	[130]
	Liver cell	[131]
	Mouse macrophage RAW264.7	[132]
	Fibroblast lung strain V79 from chinese hamster	[133]
	Leukocytes from Swiss-Webster mice	[134]
	In vivo lung cell	[135, 136]
	A549 cells and THP-1 cells	[137]
DNA Oxidation	Liver cell	[131]
	Human bronchial epithelial, alveolar epithelial and lung	[138]
	fbroblast cells	[138]
	Lung epithelial cells	[139]
Micronucleus Formation	Mouse macrophage RAW264.7	[132]
	Rat lung epithelial cells	[140]
	Normal human dermal fibroblasts (HDMEC)	[130]
	Human bronchial epithelial and mesothelial cells	[141]
	Mouse fibroblasts cell	[142]
Chromosomal Aberration	Mouse macrophage RAW264.7	[132]
	Human bronchial epithelial	[143]
	Human lymphocytes	[144]
	Mouse fibroblasts cell	[142]
Histone H2AX	Human mesothelial cells	[128]
	Chicken DT40 lymphoid cell	[145]
Mutations	Tumorigenicity in vivo	[143]
	Human bronchial epithelial and lymphoblastoid cells	[146]
	Mice lung tissue	[147]

as V(D)J recombination, which results in the production of antibodies and receptors on B and T cells. The identification of H2AX gamma is a target to identify DSB DNA breakage. However, its association with other tests for cell genotoxicity should be done in order to prevent false assertions. The detection of phosphorylated histone can be made by immunochemical assays with specifically labeled antibodies for H2AX phosphorylated histone.

Chromosomal aberrations and mutations can be detected by means of more complex methods, such as high-resolution chromosome banding techniques, chromosome hybridization (Fish, array CGH, comparative genomic hybridization) and gene sequencing. These techniques are more sensitive and require, in some cases, sequences of target genes, being of extreme importance in cases of experimental refinement, especially on topics related to the use of CNTs for transgenesis. The oxidation of nitrogenous bases of DNA molecule, which happens as a secondary response of cell oxidative stress, is verified by detection of nitroguanina-8 through specific antibodies in immunocytochemical assays. The quantification of reactive oxygen and nitrogen species (RNS and ROS) are important and complementary for the identification of genotoxicity through oxidation of nitrogenous

bases which, as previously mentioned, has a key role in breaking DNA strands. CNTs seem to induce tumorigenicity in vivo at high levels of exposure, which were demonstrated in human and mice lung cells [136, 139–140].

Micronuclei formation is characterized in the cells that have some sort of DNA damage. It consists in a small, extra nuclear body that is formed during mitosis from lagging chromosomes. In anaphase, the microtubules are not attached properly to the chromosomes, which can cause pulling in a different direction. This results in parts of the chromatids or chromosomes being broken off and enveloped as an extra nucleus in one of the daughter cells. Micronuclei can also be spontaneously formed as a byproduct of cellular defense used to remove extra chromosomes in another cell membrane, separate from the other normal chromosome. Another mechanism to micronuclei formation is by a double-strand break in the DNA, creating a separate linear fragment. As could be noticed, the observation of micronuclei formation by microscopy needs a more accurate observation by other assays in order to define it as a genotoxicity effect or a biological reponse to avoid carcinogenic effects. Micronuclei formation in response to CNT exposure were observed in many cell types of different mammalian species and most of them were associated to toxic effects of high exposure to CNTs (summarized at Table 3.2).

The future use of CNTs for development of transgenic animals needs to be clarified and all assays described above will be useful to determine its genotoxic effects. The amount and size of the CNTs nanoparticle seems to be determinant for cell fate and this must be standardized before its use as a mechanism of DNA delivery agent for generation of genetically modified mammals embryos.

3.5 Summary and Perspectives

The release of two commercial products manufactured from GMAs, as well as the large number of opportunities for different uses of these animals, point to a rapid growth for biotechnology companies. However, in order for such commercial products to reach the market effectively, there should be international normalization regulating the commerce of GMA-related products. The establishment of international standardization will bring greater security to commercial investing companies, favoring the amount of resources from the private initiative.

Specifically regarding the CNT, should the embryo transfection process using this nanomaterial be successful, similar to the successes with other cell types, this will be a nanocarrier that will allow the production of GMAs without using equipment for micromanipulation. This reduces the production cost of the GMA for two reasons: firstly, due to the costly equipment, which limits the procedure in many research laboratories. Secondly, due to the necessity of a highly specialized and trained technician to perform the embryo micromanipulation. Potentially, the use of CNTs can be far simpler and less laborious when compared to other techniques, since after its complexation with DNA, its inclusion in the culture medium used for the embryo growth in vitro could promote the production of multiple

simultaneous transgenic embryos. This opens a prospect of quick production scheduling of GMAs, which, associated with a consolidated embryo trade, could help solve some of the growing challenges of the contemporary population. However, for this technique to become a commercial reality, more research should be conducted, in order to define dose and exposure time prior to larger-scale use.

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Chapter 4
Development of Carbon Nanotubes-Based Immunogens

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Abstract The development of vaccines and the rise of the modern vaccinology are considered iconic global milestones in the history of human and veterinary medicine. The consistent use of vaccines is clearly the most cost-effective strategy both at the individual patient level as well as a public health policy. Nonetheless, we still lack vaccine solutions for many important infectious diseases and cancers. Reasons for particular failures reside mostly in the fact that classical vaccine strategies have been incapable to deliver satisfactory levels of immunogenicity and/or safety. In these cases, the development of alternative vaccine approaches appears as possible solutions to be evaluated. One such alternative strategy is the use of functionalized carbon nanotubes (f-CNT) as antigen carriers in vaccine formulations. Advantages of this kind of nanocomposite include the fact that f-CNTs are able to penetrate cells and deliver proteins within the cytoplasm, where they associate to both types of main histocompatibility complex molecules generating broad immune responses. Moreover, f-CNTs are able to modulate the immune response and function as adjuvants. There are many other important properties that make f-CNTs interesting for vaccine design, and possible drawbacks that need to be overcome before these nanocompoites can be effectively turned into usable vaccines.

4.1 Introduction

Important changes in our recent history have been largely and inherently linked to distinguishable technical and scientific breakthroughs. The discovery of electricity and radio waves, the invention of the telephone, the car, the airplane, the microchip, are just a few examples of technical and scientific advances that have deeply impacted our societies and the way we live. Nevertheless, one thing has always been central in our quest for happiness and welfare: our health. Thus, numerous discoveries in medicine and biology have also determined our paths. Examples

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include Darwin's theory of evolution; Mendel's laws of genetics; the birth of the recombinant DNA technology by Stanley Cohen and Herbert Boyer; and, more recently, the mapping of the human genome; the deciphering of gene expression regulation; and many other biomedical hallmarks.

From a more practical point of view, however, few biomedical discoveries have impacted our lives as much as the development of vaccines did. One could argue that the discovery of penicillin, and other antibiotics thereafter, was at least as important as the use of vaccination. The discovery of penicillin by Alexander Fleming, in 1928, came in a troubled interwar time, and it was determinant to drastically diminish the number of casualties during World War II. Indeed, the importance of antibiotics then and today is absolutely undisputable. Nonetheless, from a public health perspective, the use of vaccination has an unbeatable positive cost/benefit ratio when compared to therapeutic interventions. Taking vaccination against hepatitis B as an example, the prevention of the disease through the use of three subsequent doses of the vaccine, in a single individual, is one-hundred times less expensive than the treatment cost of a chronic hepatitis B condition for the same individual [1]. Moreover, the vaccination against an infectious disease has a potential amplification effect that is almost absent in the case of therapeutic treatments for the same disease. This is related to the fact that vaccination diminishes the number of individuals that are susceptible to the correlate infectious disease in a given population. As a result, the circulation of the pathogen decreases and, therefore, non-vaccinated individuals are indirectly protected against the disease; a phenomenon known as herd immunity [2]. Still, despite all benefits of vaccination, only a fraction of the existing important infectious diseases are currently preventable by the use of vaccines. Reasons for that are many, and go from technical limitations to economical explanations (reviewed in [3]). Nowadays, what drives the development of new vaccines has changed and reflects the needs of a modern global society. The new characters that play influent roles in today's vaccine design scenario are the pharmaceutical industries that dialogue directly with governments of each country. Therefore, assessments of benefit versus risk and the generated profits are now part of the development process. Although infectious diseases remain the principal targets, autoimmune diseases, cancer and even diabetes have become important challenges that can possibly be overpassed by vaccination.

4.2 The Birth of Vaccinology and Current Approaches to Vaccines

Vaccination has permanently altered the way we perceive many iconic infectious diseases such as yellow fever, tuberculosis, poliomyelitis, and smallpox, among others. When we look back in history, ages and centuries are almost always intertwined to the occurrence of large disease outbreaks; some of biblical proportions. The plague (or Black Death, a disease probably caused by the *Yersinia pestis* bacterium), for instance, is a hallmark of the medieval times, and is thought to have

reduced Europe's population by 30–60 % in the 14th century. More recently, the onset of World War I is easily and frequently associated to the 1918 flu pandemic (the Spanish flu, caused by the H1N1 Influenza virus). Smallpox is yet another infectious disease (caused by the Variola virus) that has marked historical ages. In the 20th century alone the disease caused an estimated 300–500 million deaths. It is considered by many as the most enduring and prevalent infectious disease in mankind history, as some studies estimate that the infection emerged in regions of Asia or Africa at about 10,000 years ago. Ironically, the birth of modern vaccinology is inherently linked to smallpox.

At the end of the 18th century, Eduard Jenner, a British physicist living in a farm on Gloucestershire, England, observed that milkmaids working on his property seemed to be resistant to smallpox. He also observed that most milkmaids were affected by a pustular disease manifesting on their hands and upper arms. They seemed to have contracted the disease from cows, as the animals presented similar lesions on their udders. Important to Jenner's observations was the fact that lesions in women and bovines were strikingly similar to smallpox lesions, although the disease onset was much milder in the first cases. Thus, Jenner hypothesized that the agent causing the mild disease was protecting the women against smallpox. To test his hypothesis, he collected fluid material from a lesion at the hand of a milkmaid and inoculated it in the arm of an 8-year-old boy, named James Phipps. Next, he collected fluid from a pustule on a smallpox-affected patient and challenged the boy with the infectious material. He did so twice, and the boy never developed smallpox [reviewed in 4]. Many years later, because cows were the original source of Jenner's empirical immunogen, the famous microbiologist Louis Pasteur proposed the use of the term ***vaccine*** to specify the prophylactic use of substances aiming to prevent the occurrence of an infectious disease (the word vaccine comes from *vaca*, the Latin word for cow) [5].

Although Jenner did not know at that time, what he did was to inoculate an immune competent individual with a less virulent virus closely related to *Variola virus*, named *Cowpox virus*. The generated immunity (antibodies and T cells) was able to cross-react with *Variola virus* and neutralize it, blocking the disease onset. This is the exact canonical definition of a vaccine: the use of an immunogen able to generate adaptative immune responses on an individual with the purpose of eventually neutralize a pathogenic agent related to that immunogen. Importantly, smallpox was considered eradicated from the globe in 1980, after a WHO-led vaccination campaign in which the vaccine strategy was practically identical to Jenner's approach. The main difference, however, is that the WHO vaccine was based on live *Vaccinia virus*, another attenuated virus genetically and antigenically related to the *Variola virus*, and not *Cowpox virus*. To date, the eradication of smallpox is the only man-led intervention that culminated with the eradication of an infectious disease, and is considered as one of the greatest medical achievements of all times [4, 6, 7].

Currently, all licensed human vaccines belong to one of the following classes of immunogens: attenuated vaccines, inactivated vaccines or subunit vaccines (first, second and third generation vaccines, respectively). Fourth and fifth generation

Table 4.1 Vaccine generations, vaccine instruments and diseases targeted according to each generation and instrument

Generation	Instrument	Target diseases
First	Attenuated microorganisms	Influenza, measles, mumps, polio, rabies, rotavirus, rubella, tuberculosis, typhoid, varicella, yellow fever, zoster
Second	Inactivated microorganisms	Cholera, hepatitis A, influenza, pertussis, plague, polio, typhoid
Third	Toxoid, polysaccharide, split, plasma derived, polysaccharide conjugated	Diphteria, influenza, *Haemophilus influenza,* hepatitis B, HPV, meningitis C, meningococcus, pneumococcus, tetanus, typhoid
Fourth	Subunit by recombinant DNA technology	Hepatitis B, HPV
Fifth	Reverse vaccinology	AIDS, cancer, dengue fever, malaria

vaccines combine different systems; for example, subunit vaccines and vectored vaccines made by recombinant DNA technology, or reverse vaccinology (Table 4.1). Although the final objective is always the same—to immunologically protect against an eventual infection with the correlate pathogen—the overall immunogenicity of each type of vaccine varies according the nature of its components. The next paragraphs summarize the main characteristics of each vaccine group currently licensed for human use.

4.2.1 Inactivated and Subunit Vaccines

Inactivated immunogens are composed of killed pathogens. The idea is quite straight forward: the pathogen is obtained in sufficient quantities and then inactivated through the use of different physical and/or chemical methods, including heat, ionizing radiations or chemical agents [8]. The vaccine against seasonal influenza is a good example of an inactivated vaccine in which the *Influenza virus* is grown on embrionated eggs, purified, and then inactivated by the use of either formaldehyde or beta-propiolactone (currently, an attenuated vaccine against Influenza is also available) [9]. Other examples of inactivated vaccines include immunogens against poliomyelitis (Salk), hepatitis A, rabies and the cellular pertussis vaccine (Pwc—Pertussis whole cell).

Subunit vaccines are made of isolated antigens from a given pathogen. They differ from inactivated vaccines because the whole pathogen is not present in the preparation, but only parts of it. Initially, subunit vaccines were obtained through the inactivation of whole pathogens followed by the physical separation of specific components to be added to the vaccine formulation. At the end of the 1970s, when the recombinant DNA technology became available, genes coding for antigenic proteins started to be cloned in plasmids and expressed in prokaryotic systems. After purification, these recombinant proteins could be added to the vaccine

formulation. Current systems of heterologous protein expression also include yeast and cell culture. The vaccine against hepatitis B, a vaccine against human papillomaviruses and the acellular pertussis vaccine (Pa) are examples of subunit immunogens.

Inactivated and subunit vaccines are considered inert immunogens because they are unable to replicate within the immunized individual. This feature adds safety to the formulation, as there are no living components in the vaccine; however, it also makes the vaccine less immunogenic. The reason for that lies in the fact that inert antigens are usually unable to actively penetrate the cells of the vaccinee, and therefore they have to be phagocyted by antigen presentation cells (APCs) in order to be presented to the immune system. As general rule of thumb, antigens taken up this way are associated to class II proteins of the *major histocompatibility complex* (*MHC-II*), and are presented to CD4+ T cells. This kind of antigen presentation leads to the generation of a T helper type 2 response (Th2). Additionally, inert antigens can be directly recognized by B-cell receptors (BCR) in B cells, leading to the production of antibodies. Thus, subunit and inactivated vaccines are strong inducers of humoral responses; however, because such antigens are not intensively presented to the immune cells in association to class I proteins of the *major histocompatibility complex* (*MHC-I*), they are usually poor inducers of T CD8+ cytotoxic cellular responses. Therefore, inert immunogens make up safer but less immunogenic vaccines when compared to attenuated ones [10]. In order to increase their antigenicity, intensifiers of the innate immunity responses, named adjuvants, are usually added to inactivated or subunit vaccine formulations [11, 12].

4.2.2 Attenuated Vaccines

This vaccine approach is based on the virulence attenuation of a given pathogen to a point in which it can be safely administered to immune competent subjects without causing disease, but still able to stimulate the generation of protective immune responses. There are many strategies to achieve pathogen attenuation, including repeated passages of the pathogenic microorganism in different hosts or cell cultures and, a more modern approach, the genetic and molecular modification of the pathogen through direct mutagenesis and/or recombinant DNA techniques. In any case, because the pathogen is not dead, it may be able to replicate within the immunized individual.

Due to its ability to multiply within immunized subjects, live and attenuated vaccines are able to trigger a much more diverse array of immune responses than those usually elicited by inert immunogens. One of the reasons for such differences is the fact that most attenuated pathogens are able to actively enter cells from different tissues (not only APCs) in the vaccinated individual. As a result, antigens within these cells are processed and presented to the immune system in association to *MHC-I* proteins, leading to the generation and activation of antigen-specific pools of CD8+ cytotoxic T cells. This kind of antigen presentation plus the presence

of determined co-stimulatory molecules and cytokines lead to the generation of a T helper type 1 response (Th1). Besides, the attenuated components of the vaccine may also either infect or be phagocyted by APCs, leading to presentation of MHC-II-associated peptides and activation of CD4+ T cells. Thus, the net immunity to live attenuated vaccines is usually a balanced mix of cellular and humoral antigen-specific responses [10].

There is no doubt that the multiplication competence of live vaccines makes them highly potent and immunogenic antigens; however, the same feature can also create a safety issue: they cannot be safely administered to immunocompromised individuals, pregnant women and newborns. This is due to the fact that over-replication of the attenuated vaccine in immune-incompetent subjects may induce tissue injury and disease. Furthermore, live vaccines are prone to stimulate the innate immune system in a more extensive way than inert immunogens do, mainly through the activation of multiple cell-associated *pattern recognition receptors* (PRRs). This could lead to inflammation exacerbation and the appearance of other vaccine-related adverse effects. Despite the mentioned inherent problems, most vaccines currently licensed to be used in humans belong to this class of immunogens. Examples include vaccines to yellow fever, measles, mumps, rubella, poliomyelitis (Sabin), rotavirus, tuberculosis and others [13].

4.3 Alternative Vaccine Design (Thinking Outside the Box): Antigen Delivery and the Use on Nanomaterials

As mentioned before, nowadays we have effective vaccines for many iconic infectious diseases. However, we still lack effective immunogens against a number many times larger of pathogens, either of human or veterinary importance. It is not in the scope of this chapter to discuss the reasons why many experimental vaccines have failed to deliver protection in immunized individuals. Nonetheless, there is a common saying among scientists working with infectious diseases that goes like that: “if we do not have a vaccine against a given important infectious disease is because classical vaccine strategies have been tested and failed to confer protection”. Although too generalist, some might say, the quote is possibly accurate in many, if not most cases. Indeed, for most important diseases with no current vaccine solution, experimental vaccines based either in subunit, inactivated or attenuated immunogens have already been tested and the data is published, but with no satisfactory results. In some cases, the immune responses generated in immunized individuals were not sufficient to confer protection against the disease; sometimes the vaccine induced excessive adverse events; or sometimes the strategy was technically non-feasible (for instance, not all pathogens can be safely attenuated). Thus, it has become

increasingly clear that vaccine solutions for many diseases will not be found "inside the box", but rather, through the use of alternative strategies that circumvent the current limitations of classic vaccine designs. These strategies have to be sought "outside the box".

In a provocative paper, published in 2004, Myron Levine and Marcelo Sztein [13] discussed a number of promising alternative vaccine designs that could overcome many problems of the classic vaccine strategies. Among these strategies, the authors emphasized the use of non-living antigen delivery agents. Despite their intrinsic safety, one of the major drawbacks to the use of inert antigens is the fact that they tend to be rapidly degraded after inoculation into the vaccinee. Additionally, these antigens tend to biodistribute poorly after immunization. Thus, the idea was to associate such antigens to micro-structures that would increase their stability, biodistribution, and consequently their "visibility" to the immune system. Effective non-living antigen delivery agents are particulate compounds that present hydrophobic moieties that allow their insertion into cell membranes. Moreover, they either have adjuvant properties or can co-deliver adjuvants present in the vaccine formulation [13].

Some delivery systems that present the aforementioned characteristics and have been clinically evaluated include polymeric microspheres, liposomes and virus-like particles (VLPs). However, the blooming of the nanotechnology has brought a whole new plethora of materials that intrinsically present enormous potential to be used in association to protein antigens as delivery systems. Among these, carbon nanostructures stand out. Due to their unique physical and chemical properties, these materials can be easily functionalized with different biomolecules; effectively stabilize and biodistribute them; and penetrate the cell membrane delivering the associated molecules directly into the cell cytoplasm. Furthermore, a growing body of evidence supports the idea that these materials are nontoxic if used in biocompatible amounts. In the next paragraphs, we will discuss the incipient thou increasingly robust use of Carbon Nanotubes (CNTs) as antigen delivery systems in vaccine formulations.

4.4 Carbon Nanotube-Based Immunogens

The advent of nanotechnology opened doors to resolve issues in the fields of biology, chemistry and physics. In the biological area, a myriad of applications, including drug delivery, have gained strength mainly because of singular properties of each delivery nanosystem. CNTs appear as one of the main candidates to be used as vaccines carriers in the future due to a variety of characteristics, including physical and chemical properties, together with their capacity of being functionalized with proteins, carbohydrates, lipids and DNA fragments on their surfaces.

4.4.1 CNTs Immunogenic Properties

CNTs have some advantages that allow them to be used as vaccine carriers, for example a big surface/volume ratio that make them able to carry different functional groups along its cylindrical structure; the possibility of internalization by cells through distinct mechanisms; their low toxicity, as long as inoculated in right concentrations; and biodistribution in the organism [14]. Moreover, one key attraction for the utilization of CNTs in vaccine designs is their possible role as adjuvants after administration into the body.

4.4.2 CNTs as Adjuvants in Vaccines

An adjuvant (from the latin, *adjuvare* = help) is a substance used in combination with a specific antigen to produce an immune response stronger than a response induced by the antigen alone. Adjuvants were first described by Ramon in 1924 and encompass different compounds used in vaccines (Table 4.2). As mentioned before, they are especially necessary in vaccines composed be inert immunogens. In such cases adjuvants are involved in the augmentation of the immunogenicity of the used antigens; enhancement of the immune response duration; diminishment of the required antigen doses (and the number of immunizations); stabilization of the antigens during biodistribution in the organism; and stimulation of both innate and humoral immune responses [15].

Regarding the innate immune response, it has already been demonstrated that ammonium-functionalized single-walled carbon nanotubes (SWCNTs) can successfully condense DNA CPG-motifs and achieve significant transfection in vitro to potentiate the immune response [16].

Table 4.2 Principal adjuvants used in vaccines against infectious diseases

Adjuvant	Vaccine's targeted diseases
Aluminium salt	Diphteria, hepatitis A, hepatitis B, HPV, meningococcus, pertussis, pneumococcus, polio, tetanus
Virosome/liposome	Influenza, hepatitis A
Oil-in-water emulsion/Freund's Adjuvant	Influenza
MF59™ (oil-in-water emulsion, squalene, non-ionic surfactants)	Influenza
AS04 (lipopolysaccharide—LPS—, Monophosphoryl lipid A—MPL—, Aluminium salts)	Hepatitis B, HPV
AS03 (α-tocopherol, squalene, oil-in-water emulsion)	Influenza

One important step during the initiation of the adaptative immunity is the priming of T lymphocytes (T cells). Such cells can be activated by presentation of an antigen by another cell, for example a dendritic cell, a macrophage or a neutrophil. After activation, T cells secrete cytokines to attract more cells and counter an eventual infection. T cells also interact with B lymphocytes (B cells) which are responsible for initiation of the humoral response (generation of antibodies). It has been shown that antibodies against the CD3 T cell complex, when immobilized on SWCNTs, can activate T cells and enhance presentation efficiency. Such T cell activation was quantified via secretion of Interleukin-2 (IL-2), a critical cytokine secreted by T cells in response to an activating stimulus [17]. Another work revealed that the administration of embryonic stem cells (ESC) adjuvanted with Multi Wall Carbon Nanotubes (MWCNTs) raised the therapeutic effect of the former in a colon cancer using the C57Bl/6 mouse model. Such fact was noticed after a decrease in the tumor volume and an increase in cytotoxic CD8+ T cells and T lymphocytes helper-1 (Th1-type) cytokines, including Interferon-γ (IFN-γ) and IL-2 [18].

In the human's bloodstream there is a group of proteins and glycoproteins synthesized by liver hepatocytes, blood monocytes, tissue macrophages and epithelial cells of the gastrointestinal and genitourinary tracts, named Complement. These proteins act in both innate and acquired immunity responses and their functions comprise lysing of cells, bacteria and viruses; opsonization (marking) of cells or pathogens to induce phagocytosis; binding to complement receptors on cell surfaces to induce specialized functions including inflammation; and immune clearance by removal of immune complexes from the bloodstream. The complement has three pathways that can be activated: alternative pathway, classical pathway and the lectin pathway. Regarding their activation during an infection, all pathways have the purpose to create a membrane binding complex which attack infected cells and kill the invading pathogen. It was shown that PEGylated SWCNTs induced complement activation in vitro, irrespective of the terminal end moiety of the projected PEG chains. Authors have also showed that SWCNTs failed to enhance alternative and classical pathway turnover in human serum. Therefore, SWCNTs-mediated complement activation most likely happened through activation of the lectin pathway [19].

In order to assess the cellular gene expression induced after the uptake of functionalized CNTs in immune cells, researchers have conducted microarray studies on Jurkat cells—a transformed T lymphocyte used to represent the adaptive immunity, and on THP1—a monocytic cell line that represents the innate response. Around 32,000 genes were screened and, after validation of experiments by Real Time PCR and ELISA tests, the majority of the regulated genes had their activity detected mainly on THP1 cells. Upregulated molecular pathways included IL6, CD40, dendritic cell maturation factors, tumor necrosis factor (TNF-α), Nuclear Factor Kappa Beta (NFκB) signaling and T helper lymphocyte 1 chemokine pathways, all usually activated during acute inflammatory processes and pathogen clearance which are necessary in vaccination responses [20].

According to some researches, when nanoparticles reach a physiologic system, for example the human plasma, they selectively absorb some molecules to form a biomolecular corona around the particle. This corona may persist depending on the degree of hydrophobicity of the particle. Lipids attached to the corona may facilitate the penetration of nanoparticles through cells and could confer a new identity to the nanocomposite. Some authors say that this new identity could represent a novel type of molecular pattern to be recognized by the immune system and thus elicit immune responses. Once added o a vaccine formulation, these NAMPs (Nanoparticle-associated molecular patterns) could work as adjuvants and help initiating the innate response [21]. In fact, a lot of attention has been drawn to modeling nanoparticles, in particular CNTs, in order to create the perfect delivery system that could also be capable of acting as an adjuvant. Functionalization, purity, diameter and length, among other features, must be taken into account when designing CNTs to work as delivery systems.

4.4.3 CNTs Penetration in Cellular Membranes

The use of CNTs as vaccine carriers raises the following question: how are CNTs internalized into cells to deliver the antigen? Some researchers argue that CNTs penetrate like a needle though plasma membranes, like a simple diffusion or some type of passive transportation without the requirement of energy [22, 23]. Others suggest that cellular uptake occurs via endocytosis, an energy-dependent pathway which confines the SWCNTs into clathrin-coated vesicles originated from the membrane [24]. Villa and collaborators showed that SWCNTs conjugated with Wilm's tumor protein (WT1) were internalized and accumulated within dendritic cells through macropinocytosis. Once internalized, the carried proteins were subsequent shuttled to MHC class II compartments [25]. One research group has done a computer simulation of how CNTs penetrate the cell membrane and, according to them, our cellular membranes can intake both functionalized and pristine CNTs. The in silico simulations revealed that larger CNTs cause greater membrane perturbation and are subjected to rotation and translation during their entrance. They described an interesting and possible one-step pathway in which the insertion of the CNT into the hydrophobic core of the membrane occurs with water molecules being expelled during the process [26].

What must be taken into account is that CNTs cell internalization depends on many factors like: Different temperatures; cell membrane compositions; cell types; CNTs diameter, length and purity; the presence or not of functionalized groups; and the method of functionalization are all crucial characteristics that interfere in the way CNTs penetrate cells. Therefore, more studies are needed to compare each of these factors and to finally establish, perhaps, not a main pathway, but the most suitable pathway concerning the goal of each experiment.

One issue that has also not been elucidated so far is the CNTs fate after cell loading. Studies point to the possibility of cell-released vesicles carrying CNTs to extracellular fluids and their delivery to naive cells. One group has demonstrated that CNTs expelled by stressed macrophages and endothelial cells could be taken up by other cells, including phagocytic ones. Thus, it could be argued that intercellular transportation of CNTs might be another pathway to deliver antigens and also to stimulate more cells of the immune system [27].

4.4.4 CNT-Based Experimental Vaccines

Perhaps one of the most intriguing and attractive biological application of CNTs is their use in the development of vaccines against infectious diseases. Due to their high surface/volume ratio, the possibility of CNT functionalization with antigenic peptides to stimulate the immune response represents an alternative to classic strategies such as inactivated and subunit vaccines or attenuated pathogens. Indeed, CNTs carrying peptides derived from pathogens or tumors have been shown to be immunogenic and protective in many experimental settings and animal models.

Pantarotto and colleagues were among the first to test the feasibility of a CNT-based vaccine-delivery systems using a *Foot-and-mouth disease virus* (FMDV) model. The authors constructed SWCNTs conjugated with a fragment from the immunogenic VP-1 viral protein. The immunogenicity of the vaccine was tested after immunization of BALB/c mice and observation of the positive reaction between the generated antibodies and VP-1 peptide fragments as solid phase in an ELISA test. The robust induction of anti-VP-1 specific antibodies in vaccinated animals demonstrated the NTC ability to keep the peptide's original three-dimensional conformation [28]. Moreover, no particular responses against the SWCNTs were verified during the experiments, which suggests the possible absence of detectable toxicity of these particles.

Yandar and colleagues functionalized MWCNTs with the N-terminal part (residues 21–42) of the Apical Membrane Antigen-1 (AMA-1) from *Plasmodium vivax*, a causative agent of Malaria. This construction was tested in BALB/c mice and the immunization induced the production of antibodies capable of impeding the infectious agent from invading erythrocytes. Results also showed that the antibodies generated after immunization were capable to reduce the parasitemia of *Plasmodium berghei*—another Malaria-like agent—in mice [29].

In yet another study, researchers coated SWCNTs with tuberculin purified protein derivative (PPD), a mixture of antigens derived from a culture of *Mycobacterium tuberculosis*—the pathogen responsible for tuberculosis, in order to evaluate the immune response upon mice immunization. The resultant cytokine profile indicated a prevalence of Th1 responses due to the production of IFN-γ and IL-12. Besides, low levels of IL-5 and IL-10 were also detected, which denotes a low stimulation of Th2 response [30].

Silvestre and collaborators [31] developed an immunogen for *Anaplasma marginale* utilizing carbon nanotubes as carriers in a murine model. Mice immunized with NTC-associated recombinant *Anaplasma* surface proteins (rMSP1a) produced high levels of anti-rMSP1a IgG, demonstrating that rMSP1a functionalization to carbon nanotubes did not interfere with protein immunogenicity. Immunization with MWNT-rMSP1a induced significantly higher percentages of CD4+CD44+ and CD4+CD62L+ lymphocytes, higher levels of TNF-α, and a higher proliferative rate of splenocytes when compared to mice immunized with rMSP1a alone.

Concerning vaccines against cancer, Villa and colleagues worked with the Wilm's tumor protein (WT1) which is up-regulated in many human leukemias and kidney cancer [26]. They conjugated this protein to SWCNTs and incubated them with dendritic cells. The construct was internalized through a macropinocytosis mechanism and elicited CD4+ T cell IFN-γ dependent responses in human lymphocytes. Additionally, SWCNTs conjugated to peptides induced a higher IgG-based humoral response, in BALB/c mice, after four administrations using TiterMax, a commercial adjuvant.

The use of CNTs as a vaccine delivery system or as an adjuvant is still incipient. A few pioneering works have been published so far, but they have clearly demonstrated the enormous applicability of this new tool to the vaccinology field. Indeed, when we simply compare cell-based responses in Balb/C experimentally immunized either with a subunit immunogen against Dengue or with the same immunogen functionalized to CNTs, the difference in favor of the second is intense (Fig. 4.1), further showing the incredible potential of CNT-based immune reagents [32].

Obviously, as any new approach, this one still have to go through intense public and scientific scrutiny before they could be considered fit to undergo clinical trials, especially considering that CNTs toxicity is still an issue for many researchers and public health agents.

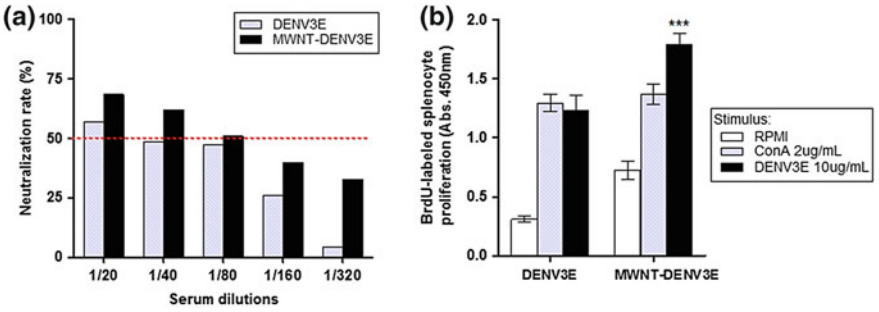

Fig. 4.1 Evaluation of Humoral and Cell-mediated responses of Dengue virus vaccine based on carbon nanotubes. **a** Plaque reduction neutralization test to dengue virus, were vaccine group show neutralizing response in greatest serum dilutions (1/80), in compare to the protein group. **b** Lymphocyte proliferation by BrdU-labeling [32]

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Chapter 5
Fullerene-Derivatives as Therapeutic Agents in Respiratory System and Neurodegenerative Disorders

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Abstract Since the discovery of fullerenes in 1985, this class of spherical or ellipsoid molecules made entirely of carbon atoms has attracted great interest in the biological field because of its unique physical and chemical properties. However, due to the insolubility in polar solvents and the aggregation properties, the biological applications of these molecules have been restricted. To overcome this problem, water-soluble derivatives of fullerenes have been developed. Fullerenes and fullerene-derivatives react readily with electron rich species as radicals, and because of this property they have great potential to be used as antioxidants. Oxidative stress occurs when the cellular production of reactive oxygen species exceeds the capacity of antioxidant defenses within cells. In humans, oxidative stress is associated with the development and progression of several pathologies, including neurodegenerative and pulmonary diseases. This chapter brings together the current understanding of how fullerene-based materials may be used as therapeutic agents and contributes to reduce oxidative stress in respiratory system diseases and neurodegenerative disorders.

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5.1 Introduction

5.1.1 Oxidative Stress and Disease Development

Oxidative stress is a general term used to describe disturbances in the normal redox state of cells that occur when the cellular production of reactive oxygen species (ROS) exceeds the capacity of antioxidant defenses within cells [1]. The oxidative stress leads to damage of all components of the cell, including deoxyribonucleic acid (DNA), proteins, and lipids affecting the normal mechanism of cellular signaling. In humans, oxidative stress is associated with the development and progression of several pathologies, including cancer [2], Parkinson's and Alzheimer's diseases, amyotrophic lateral sclerosis [1], atherosclerosis, chronic congestive heart failure (CHF), myocardial infarction [3], asthma, acute lung injury/acute respiratory distress syndrome, sepsis-related lung injury, fibrosis and emphysema chronic obstructive pulmonary disease [4].

ROS can exert beneficial or deleterious effects that depend on the type and concentration of ROS produced in the organism [5]. Physiological levels of ROS can act as signaling molecules to drive several cellular functions activating redox-sensitive kinases, phosphatases, and transcriptional factors responsible for modulating gene transcription and affecting cell survival. On the other hand, high levels of ROS can cause significant damage to cellular proteins, membranes and nucleic acids, leading to cellular dysfunction and death [5–7].

This chapter summarizes fullerenes chemical properties (Sect. 5.1.2) and their antioxidant effects (Sect. 5.1.3). Sects. 5.2 and 5.3 describe fullerene-based materials effects in neurological and respiratory diseases, respectively. Sect. 5.4 gives a summary and discusses the perspectives for the use of fullerene and fullerene-based materials on both systems.

5.1.2 Fullerenes Chemical Properties

Fullerenes represent a class of spherical or ellipsoid molecules made entirely of carbon atoms in a cage-like structure composed of pentagonal and hexagonal faces. Fullerenes were discovery in 1985 by Harry Kroto, Richard Smalley and Robert Curl [8]. Further in 1996, this discovery led to the Nobel Prize of Chemistry.

The number of carbon atoms in each fullerene cage can vary, creating the possibility of numerous new structures, that are represented by the formula C_n (n = number of carbon atoms present in the cage). Since C_{60} discovery, several isomeric forms are confirmed to exist as stable clusters including C_{70}, C_{76}, C_{78}, C_{82}, C_{84}, C_{90} and C_{96}, however C_{60} is the most abundant and well characterized structure (Fig. 5.1). According to Euler's theorem, a closed structure can be construct with 12 pentagons, by this means all fullerenes consist of 12 pentagonal faces and a varying number of hexagonal faces, following the general formula C_{20+2n}.

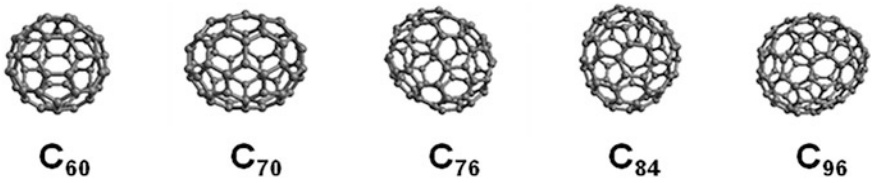

Fig. 5.1 Some fullerene isomeric isoforms [9]

The C_{60} plays significant features as all the 12 pentagons are isolated by hexagons and as it posses different types of bonds: the 6–6 junctions shared by two adjacent hexagons, also known as "short bonds" and the 5–6 junctions present in the juncture of a hexagon and a pentagon, the "long bonds" [10].

As a result of its bond-alternated structure, fullerene C_{60} should not be considered as a real aromatic compound but as a strained, electron-deficient polyolefin. The pyrimidalization of the sp^2-hybridized carbon atoms configure a non-fully planar surface without possibility of electronic ring current. The excess of strain on the surface of C_{60} is responsible for its high reactivity compared to other forms of carbon, because as they react, the functionalized fullerene carbon atoms change their hybridization from a trigonal sp^2 to a less strained tetrahedral sp^3. This particular characteristic of fullerenes makes them reacts readily with electron rich species as radicals, various nucleophiles, and carbenes and they also participate in many thermal cycloadditions reactions, including Diels-Alder [10]. Therefore, this rich chemistry allows a wide range of application for specific proposes [11].

In the biology field, the unique physical and chemical properties of these molecules led many scientists to predict several applications [12–17]. However, due to the insolubility in polar solvents and the aggregation properties, the biological applications have been restricted [18]. Indeed, fullerenes are hydrophobic molecules most common soluble in benzenes, naphtalenes and alkanes [19].

In order to overcome this disadvantage new approaches have been developed to provide its water solubility. Among these approaches we can cite: (i) chemical modifications of the fullerene cage by attachment of several functional groups. The most common chemical groups used are hydroxyl [20], carboxyl [21, 22] and amide [23]; (ii) covering the core of fullerenes with modifying agents such as surfactants, calixarenes [24] and cyclodextrins [25].

5.1.3 Antioxidative Properties of Fullerenes

Several lines of evidence support the fact that fullerenes are powerfull antioxidants, acting as a radical sponge [26]. The fullerene's antioxidant property is based on the fact that fullerenes possess an energetically low-lying, threefold-degenerate lowest unoccupied molecular orbital (LUMO) which can easily take up to six electrons, making an attack of radical species highly possible [27]. In addition, it is clear that nano-size

(diameter of ~1 nm), three-dimensionality and physical properties make the fullerenes and its derivatives, like fullerenol ($C_{60}(OH)_x$), very appealing subjects in medicinal chemistry [28, 29]. Due to these features fullerenes were considered to be the world's most efficient radical scavenger and were described as radical sponges [26].

The protective activity of fullerenes and derivatives to inhibit the chain reaction of lipid peroxidation is based on their capability to react with oxygen radical species such as superoxide, hydroxyl and peroxyl radicals, which attack lipids, proteins, DNA, and other macromolecules, which would lead to cell death [27, 29, 30].

5.2 Fullerene-Based Materials as Neuroprotective Agents

Oxidative stress is involved in the pathogenesis of a number of neurological disorders, including ischemia, and neurodegenerative diseases [31–33]. These states are associated with progressive impairment of mitochondrial function, increased oxidative damage and altered activities of antioxidant defense system. Toward the goal of reducing the oxidative stress in brain injuries, efforts have been focused on the development of new neuroprotective agents [34, 35]. Fullerene-based materials have been exploited for its purpose as excellent antioxidants, therefore possessing a broad spectrum of neuroprotective abilities [12, 36].

One of the most promising and studied classes of water-soluble fullerene-derivatives is carboxyfullerenes, and its neuroprotective efficacy has been established in several models. Dugan et al. [12] using cortical cell cultures exposed to excitotoxic and apoptotic injuries have demonstrated that malonic acid derivatives of C_{60}, ($C_{63}((COOH)_2)_3$) decreased excitotoxic neuronal death in cortical neurons exposed to N-methyl-D-aspartate (NMDA), α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA), or oxygen-glucose deprivation. Intriguing in vivo data by the same group showed that infusion of carboxyfullerenes in a transgenic mouse model for familial amyotrophic lateral sclerosis delayed onset of symptoms and death [12]. Remarkably, carboxyfullerene-treated mice showed improved functional performance when compared to saline treated mice. In addition, these authors found that carboxyfullerenes can also reduce apoptosis induced by serum deprivation or by the Alzheimer disease amyloid peptide (Aβ1–42) [12]. Further insight into the use of fullerene derivatives for the treatment of Alzheimer's disease came from the work of Makarova et al. [37]. In this study, the authors performed intrahippocampal microinjections of beta-amyloid peptide 25–35. Importantly, administration of aqueous colloidal solution of C_{60} prevented formation of beta-amyloid 25–35 deposits in hippocampal pyramidal neurons in vivo and neurodegeneration.

Focal cerebral ischemia is associated with a massive production of intravascular free radicals [38]. A variety of free radical removers and antioxidants are known to ameliorate the injury associated with ischemia reperfusion [39]. Huang et al. [40] investigating the effects of hexasulfobutylated C_{60} on the total volume infarct size elicited by focal cerebral ischemia in vivo reported beneficial effects in reducing

volume infarct size. Similar findings were reported by Lin et al. [41] in another model of focal ischemia-reperfusion injury in rat brain. In this study, carboxyfullerene was administered by intravenous injection or intracerebroventricular infusion to rats 30 min prior to the induction of transient ischemia-reperfusion. While no protection of cortical infarction was observed after intravenous injection of carboxyfullerene, intracerebroventricular injection significantly decreased the size of infarcted area and attenuated oxidative injury caused by transient ischemia-reperfusion. It is worth mentioning that these protective effects of carboxyfullerene were also associated with adverse behavioral changes, indicating that these fullerene-derivatives may present undesired side effects when administered in vivo [41]. This finding raises questions regarding toxicity issues related to C_{60} and its use in nanomedicine. In fact, in vitro experiments performed by Zha et al. [42] support the notion that fullerene derivatives present a dual effect, depending on the concentration used. In this study, it has been shown that, at low concentrations, water-soluble polyhydroxyfullerene significantly increased hippocampal neuronal viability and protected neurons from oxidative damage, while, at high concentrations, this fullerene-derivative decreased hippocampal neuron viability and induced apoptosis. Thus, these data highlight the importance of more fundamental research to be carried out, in order to better understand these concentration-dependent dual actions of fullerene derivatives and the underlying mechanisms.

The in vivo ability of carboxyfullerenes to exert neuroprotection was also evaluated in a study conducted by Lin et al. [43]. In this work, the authors used intrastriatal injection of iron to induce degeneration of the nigrostriatal dopaminergic system. Oxidative injury and decreased dopamine content in striatum were observed 7 days after iron infusion. The co-infusion of carboxyfullerene prevented the iron-induced oxidative injury. In the nigrostriatal dopaminergic system the authors did not observe toxic effects of carboxyfullerene.

Among the fullerene derivatives, the polyhydroxylated $C_{60}(OH)_{22}$ and malonic acid $C_{60}(C(COOH)_2)_2$ derivatives were show by Lao et al. [44] to protect against nitric oxide (NO)-induced apoptosis in sodium nitroprusside-treated rat cerebral microvessel endothelial cells (CMECs). This finding supports a potential application of fullerene derivatives in the treatment of NO related disorders.

Most studies attributed the protective effects of fullerene derivatives at least partly to its free radical scavenger properties [11]. Consistent with this idea, Ali et al. [45] proposed that C_3 malonic acid C_{60} derivative acts as a superoxide dismutase mimetic, and they demonstrated its presence in mitochondria, a major site of reactive oxygen species production in the cell. Based on the fact that fullerene derivatives are able to penetrate the blood-brain barrier and preferentially localize to mitochondria [46], Cai et al. [47] hypothesized that these compounds could be used as neuroprotective agents for treating Parkinson's disease. In this study, the authors treated human neuroblastoma cells with MPP1 (1-methyl-4-phenylpyridinium), a cellular model of Parkinson's disease, and evaluated the protective effects of $C_{60}(OH)_{24}$ on MPP1- induced mitochondrial dysfunction and oxidative stress. These results revealed that polyhydroxylated fullerene derivative is a powerful radical scavenger that protects the mitochondria. Thus, this finding is in agreement with previous

reports that support the role of fullerene-derivatives as potential neuroprotective agents capable of protecting against disorders associated with mitochondrial dysfunction, such as neurodegenerative diseases [48].

5.3 Fullerene-Based Therapeutics in Respiratory System

5.3.1 Reactive Oxygen Species in the Lungs

The reactive oxygen species (ROS), mainly derivate from oxygen and nitrogen, are continuously produced in several tissues and cells. At low concentration, ROS play an important physiological role, such as microbial defense system, vascular tonus control, cell signaling, and others [49, 50]. The excess is removed by enzymatic and non-enzymatic ways. However, when the production of ROS is high, the mechanisms of control fail, or there is imbalance between production and degradation, the organism suffers with oxidative stress that is a potential cause of damage [51].

Although oxygen is fundamental to life, high concentrations can exceed physiological limits and be dangerous to the cells [52]. Considering that the respiratory system is directly exposed to very high amounts of the oxygen [53–55], it is very important for the organ to possess defenses against oxidative attacks. Moreover, the lungs receive entire cardiac output, being the main target of systemic ROS. Thus, antioxidant defenses in lungs are primordial for the balance of ROS [52].

A wide variety of respiratory system cells can produce ROS, such as endothelial cells, Clara cells, alveolar type II cells (type II pneumocytes), neutrophils and macrophages. The process of production is the same of the other cells and systems [56]. The main enzymes involved in ROS synthesis are nicotinamide adenine dinucleotide phosphate—oxidase (NADPH oxidase), myeloperoxidase (MPO), xanthine oxidase and coupled and uncoupled nitric oxide synthases (NOS), and for ROS degradation, the most important enzymes are superoxide dismutase (SOD), catalase and glutathione peroxidase (GPx) [57].

Once produced, ROS can act in the respiratory system mainly in tracheal and bronchiolar smooth muscle and in pulmonary blood vessels. The primary effect of ROS in lungs is airways hyperresponsiviness to contractile agents [50, 51]. In pulmonary blood vessels the effect of ROS is variable. It is largely accepted that oxidative stress in lung can lead to vasoconstriction due to superoxide anion (O_2^-). However, in cases where there is the production of high amounts of nitric oxide (NO) it is observed pulmonary blood vessels hyporresponsiviness to contractile agents [58]. In both cases, pulmonary hypertension due to hypoxic vasoconstriction is observed [59, 60].

Notably, the lungs have particular mechanisms for its defense against massive production of ROS. Epithelial fluid is rich in glutathione (GSH), an important human pulmonary antioxidant. This substance is able to bind a free iron ion and other metals, and to inhibit synthesis of ROS through the Fenton reaction [61].

There are others antioxidants like water and fat-soluble vitamins on the mucosis secretion of airways that prevent changes on mitochondrial oxidant status. In addition, endogenous antioxidants enzymes such as SOD, catalase and others related with GSH metabolism—and exogenous antioxidants—like vitamins C and E, and N-acetylcistein may also help to prevent the imbalance between oxidant and antioxidant status [51].

In this context, new antioxidants can arise for the treatment of pulmonary diseases associated with the production of ROS. The polyhydroxylated fullerene (fullerenol) is a powerful antioxidant that can be useful because of its specific structure, electronegativity [28] and nanoscale [62], which drives its role like scavenger of ROS [55].

5.3.2 Fullerene-Based Materials and the Respiratory System

Several respiratory diseases, such as asthma, acute lung injury/acute respiratory distress syndrome, sepsis-related lung injury, fibrosis, emphysema chronic obstructive pulmonary disease and hypovolemic shock are associated with an increased production of ROS.

Hypovolemic shock after exsanguination induces increase of ROS production [63]. Subsequently, afferent C-fibers are activated [64] producing tachykinins release and noncholinergic airway constriction. Lai and Chiang [28] evaluated whether fullerenol changed the pulmonary function and whether fullerenol attenuated the exsanguination-induced bronchoconstriction in guinea-pigs. Firstly, they observed that fullerenol does not usually cause alteration in pulmonary function unless it is administered via an intratracheal route in a high dosage (2 mg/kg). The reason for this slight alteration in respiratory function with a high dosage of intratracheal fullerenol was not clear. A possible explanation is that at this dose fullerenol can generate more ROS and cause a mild airway constriction [28]. In a second time they showed that fullerenol ameliorated the exsanguination-induced bronchoconstriction. The antioxidant action of fullerenol probably antagonized the exsanguination-induced increase in ROS production, thereby decreasing the activation of afferent C-fibers, tachykinin release, and airway constriction following exsanguinations in those animals [28].

Lung transplantation is an important way to treat a variety of end-stage lung diseases. Despite this fact, a central problem following this procedure is ischemia-reperfusion injury. This damage happens when blood supply returns to the lung after the completion of the implantation procedure [65]. The alveolar oxygen represents a risk for generation of ROS and continuous oxygen supply can increase ischemia-reperfusion injury [66]. Antioxidant therapy arises like a potential approach to attenuate this injury. Chen et al. [67] carried out experiments in ischemia-reperfused lungs to examine whether fullerene derivatives [$C_{60}(OH)_{7\pm2}$] can protects against injury induced by ROS. When applied in lungs during

ischemia-reperfusion procedure, sodium nitroprusside (SNP) increased pulmonary artery pressure and capillary filtration pressure. The fullerene derivative [$C_{60}(OH)_{7\pm2}$] (10 mg/Kg) attenuated SNP-induced lung injury during ischemia-reperfusion. Chen et al. [67] also investigated whether fullerene derivatives [$C_{60}(OH)_{7\pm2}$] protects against oxidative stress in a murine macrophage RAW 264.7 cell line. They used SNP and hydrogen peroxide (H_2O_2) to enhance NO and oxidants, respectively. They showed that $C_{60}(OH)_{7\pm2}$, in a concentration-dependent manner (10–50 μM), inhibited the increase in ROS production, and the decrease in mitochondrial membrane potential and cell viability induced by SNP and H_2O_2. Based on the above results, Chen et al. [67] suggested that due to their antioxidant characteristics, fullerene derivatives [$C_{60}(OH)_{7\pm2}$] can be used to prevent ischemia-reperfusion injury that follows lung transplantation. They additionally, hypothesized that fullerene derivatives could be potentially useful for the treatment of ROS-induced pulmonary diseases such as sepsis-related lung injury, acute respiratory distress syndrome, fibrosis, emphysema, and asthma.

Inflammation plays a central role in eliminating pathogens and promoting repair of injured tissue. The resolution of this process is fundamental, since excessive or persistent inflammation can lead to tissue damage and exacerbation of diseases, including inflammatory lung diseases, such as acute lung injury [49], chronic obstructive pulmonary disease [68] and silicosis [69]. Exposure to quartz particles leads to ROS production, which leads to lung inflammation. Additional injuries can occur in response to the arrival of inflammatory cells [69]. The quartz-induced inflammation is characterized by neutrophilic inflammation in rodents [70]. Roursgaard et al. [71] studied neutrophilic lung inflammation induced by quartz and evaluated whether pre-treatment with fullerenol [$C_{60}(OH)_{20\pm2}$] at low doses can attenuate this pulmonary disease. They hypothesized that fullerenol would be able to neutralize ROS and thereby attenuate inflammation induced by ROS. Quartz induced a marked inflammation characterized by increase in the number of neutrophil in bronchoalveolar lavage (BAL). Interestingly, fullerenol (0.2, 2.0 and 20 μg) given to mice before quartz instillation decreased neutrophilic lung inflammation in a dose-dependent manner. As a control, mice were exposed to increasing doses of fullerenol alone given intratracheally. The BAL data indicated that only at the highest dose (200 μg), fullerenol induced lung inflammation primarily caused by neutrophils infiltration. This increased number of neutrophils was associated with an increased level of macrophage inflammatory protein 2 (MIP-2), which is a very important neutrophil attractant chemokine. At the doses of 0.02, 0.2, 2.0 and 20.0 μg, fullerenol alone was without inflammatory effect. The authors suggested that this compound has an anti-inflammatory effect at low doses but a pro-inflammatory effect at higher doses and concluded that quartz-induced neutrophilic inflammation in the lungs can be attenuated by administration of the water-soluble polyhydroxylate fullerene derivative (fullerenol) at low doses [71].

Similar results were found by Sayes et al. [72] that showed that intratracheal instillation of low doses (0.2 mg/Kg) of fullerenol [$C_{60}(OH)_{24}$] did not induce neutrophilic inflammation, whereas high doses (1.5 mg/Kg) of the same compound produced marked inflammation in rats.

Doxorubicin, an anthracycline antibiotic, is considered as a broad spectrum antineoplastic agent, which represents one of the most effective chemotherapy agent currently in use for cancer treatment [55, 73]. Doxorubicin antitumor effects include mechanisms related to alterations of DNA and production of free radicals [74]. The most frequent investigated effect of this agent at a molecular level is its tendency to generate large amounts of ROS [54]. In addition, doxorubicin inhibits ROS neutralizing enzymes [54, 55, 75]. A variety of tissues like heart, liver [76], lung [54], testes and kidneys [55], are also affected by this agent. Due to doxorubicin's toxicities, its use in chemotherapy has been largely limited [55, 73, 74]. It is believed that oxidative stress and formation of free radicals play a crucial role in doxorubicin's toxic mechanism. Thus, the use of antioxidants as protective agents could be a potential solution for doxorubicin-induced toxicity [77] in a variety of tissues.

Injac et al. [54] investigated the potential protective effect of 100 mg/kg of fullerenol [$C_{60}(OH)_{24}$] on the lungs of Sprague-Dawley rats with mammary neoplasm after doxorubicin (8 mg/kg). This study indicated that doxorubicin treatment markedly impairs pulmonary function and that pre-treatment with fullerenol (100 mg/kg) prevented this toxicity in rats via inhibition of oxidative stress. In that paper, the authors showed that fullerenol normalized the activity of antioxidants enzymes (catalase, glutathione reductase, GPx, SOD) and lipid peroxidation to the control levels.

The above results are in accordance with Srdjenovic et al. [78], which showed that fullerenol [$C_{60}(OH)_{24}$] inhibited doxorubicin-induced toxicity in lungs, kidney and testes of Wistar rats. They analyzed lipid peroxidation and antioxidant enzymes activity (catalase, glutathione reductase, GPx, glutathione S transferase and SOD) and found that fullerenol in a dose of 100 mg/kg inhibited the oxidative stress in kidney, testes and lungs 2 days after doxorubicin treatment. Moreover, the results with the dose of 50 mg/kg also indicated that, at the period of 14 days after doxorubicin, fullerenol [$C_{60}(OH)_{24}$] induced an important tissue-protection in the analyzed organs.

Vapa et al. [55] also showed that fullerenol [$C_{60}(OH)_{24}$] (50 and 100 mg/kg) had protective effect against lipid peroxidation in testes, kidneys and lungs after the application of doxorubicin (10 mg/kg) in rats.

The toxicity of fullerene derivates in vitro has been investigated in cell lines. Cytotoxicity effects have been related, mainly to high concentrations, but a correlation between in vitro and in vivo toxicity measurements was not observed [72]. In addition, the underivatized fullerene (C_{60}) is 3–4 orders of magnitude more toxic to human dermal fibroblasts, lung epithelial cells and normal human astrocytes when compared to a fully derivatized, highly water-soluble derivate, fullerenol [$C_{60}(OH)_{24}$] [79].

Some pulmonary diseases, such as asthma and chronic obstructive pulmonary disease, require treatment by inhalation. This administration route provides rapid delivery of drugs to the lungs for local treatment of respiratory diseases, avoiding systemic side effects and evading liver first-pass effects [80, 81]. At this regard, the effect of fullerene derivatives like fullerenol given by inhalatory route deserves further investigation. Sayes et al. [72] carried out a study, where they assessed the

lung toxicity of intratracheal instilled fullerene (C_{60}) and fullerenol [$C_{60}(OH)_{24}$] in rats. They applied these compounds at the doses of 0.2, 0.4, 1.5 and 3.0 mg/kg and analyzed the effects 24 h, 1 week, 1 month and 3 months after the instillation and compared with α-quarts-induced lung disease. Exposures to fullerene (C_{60}) and fullerenol [$C_{60}(OH)_{24}$] suspensions did not produce any significant adverse pulmonary effects. Results from the BAL fluid and cell proliferation evaluations demonstrated that pulmonary exposures to α-quartz particles, specially at the higher doses (1.5 and 3 mg/kg), produced significant adverse effects as compared to the controls in pulmonary inflammation (neutrophils number and histology), cytotoxicity (dehydrogenase latic, alkaline phosphatase and total protein content) and lung parenchymal cell proliferation indices. In contrast, both compounds produced transient and reversible inflammation, similar to exposures to Milli-Q water (vehicle), showing that inflammatory response was likely related to the effects of the instillation procedure.

Xu et al. [62] also measured fullerenol [$C_{60}(OH)_{22–24}$] effects after intratracheal instillation in Sprague-Dawley rats at the doses of 1, 5 or 10 mg/rat. Exposures to 1 mg per rat did not induced adverse pulmonary toxicity, while 5 and 10 mg per rat induced cell injury effects (increased pulmonary vascular permeability and increased activity of dehydrogenase lactic and, alkaline and acid phosphatases), oxidative/nitrosative stress (increased lipid peroxidation and NOS activity and, reduced glutathione content and SOD activity) and inflammation (increased TNF-α, IL-1β, IL-6 and neutrophils number on BAL and blood). Their results showed that only the highest dosages (5 and 10 mg per rat) of fullerenol [$C_{60}(OH)_{22–24}$] produced pulmonary inflammatory response. The aggregation of the fullerenol particles in these high-doses may lead to inflammation and be responsible for the pulmonary toxicity. Thus, they concluded that fullerenol particles would have a potential risk for producing pulmonary toxicity and suggested the use of fullerenol as an inhaled drug in short-term and low doses.

The literature about fullerenol value on respiratory system is poor. Therefore, more investigations are essential because the potential use of this compound to improve and treat lung diseases is considerable.

5.4 Summary and Perspectives

The value and applicability of fullerene and fullerene derivatives (fullerenol) for the treatment of neurological and respiratory disorders that are associated with oxidative stress is undoubted. However, caution must be taken with dose and via of administration. Moreover, the difficulty of interpretation and extrapolation of in vitro toxicity measurements to in vivo effects, highlight the complexity associated with probing the relevant toxicological response of fullerene nanoparticle systems. Therefore, more studies are necessary to elucidate the most suitable dosage and via of administration of fullerenol that induce the best results.

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Chapter 6
Study of Carbon Nanostructures for Soil Fertility Improvement

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Abstract Keeping organic matter in the soil is one of the most promising carbon capture and storage methods. Soil organic matter has the longest soil persistence and cause soil amelioration. The "*Terra Preta de Índio*" (Amazonian Dark Earth) is an anthropogenic Amazonian soil that provides a potential model for such organic matter soil storage, generating a sustainable land-use system that is highly efficient even in the hot and humid tropical regions. The large amount of carbon-based materials in these soils is responsible for their unusually high fertility over long periods of usage. In this chapter, by applying materials science tools, including

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scanning and transmission electron microscopy, energy dispersive X-ray, electron energy loss spectroscopy and Raman spectroscopy, we show that these millenary carbon materials exhibit a complex morphology, with particles ranging in size from micrometers to nanometers, from the core to the surface of the carbon grains, and are rich on specific elements that are important for fertility and carbon stability. Specifically, calcium and oxygen are abundant in the whole carbon structure, and the role of these elements on carbon stability is studied theoretically. From one side, our results might elucidate how nature solved the problem of keeping high levels of ion exchange capacity in these soils. From the other side, morphology and dimensionality are the key issues in nanotechnology, and the structural aspects revealed here may help generating the "Terra Preta Nova" (New Black Earth), effectively improving world agriculture and ecosystem sustainability.

6.1 Introduction

6.1.1 Soil Amelioration and Carbon Storage

Two important concerns of our present society are carbon storage and amelioration of soil recalcitrance and fertility. Although apparently uncorrelated, these two problems can be treated within a unified approach, since soil biogeochemistry can be tuned significantly by the addition of organic matter. Therefore, the unified direction can be achieved by the storage in the soil of carbon residues generated by human activity, aiming at soil amelioration for enhancing and balancing food or biofuel production and waste. The model of slash and burn is the outlook for the stock of carbon from the atmosphere through the burning of organic matter under controlled conditions [1–3].

The presence of stable carbon structures in the soil, usually referred to as pyrolysed biomass, or black carbon or biochar [3–7], offers improved soil productivity [3, 4, 8], further increasing biomass production. It has also been claimed that sinking carbon via low-temperature pyrolysis in soil is more efficient than the storage of carbon in plants and trees [3–5], because it increases the carbon mean residence time.

6.1.2 Tropical Region and a Sustainable Model

The use of biochar to improve soil recalcitrance and productivity can generate special impact in the lifestyle of people living in the tropical regions, which is home

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to more than 4 billion people, many of which reside in developing countries. Although soils in the tropics are generally characterized by low nutrient retention due to heavy rains and high temperatures, deforestation leading to desertification [9, 10], there are interesting and rather exotic exceptions, which are sites of highly fertile areas in the Amazonian region. These areas, known as *Terras Pretas de Índio* (TPI), or Amazonian Dark Earths, are always found containing pieces of ceramics, which indicates the presence of ancient civilizations living in these areas [11]. The origin of this special soil is considered to be anthropogenic, from Pre-Columbian civilizations [12], ranging from 600 to as much as thousands of years [13], in an accumulation and burning process of organic matter, intentionally or as a by-product of the occupation by ancient civilizations.

The *Terras Pretas de Índio* (TPIs) are more common in sites at the Amazon basin [5, 14–16], 20 ha on average [16], but they can also be found throughout the humid tropics and in other regions of South America and Africa [17, 18]. The discovery of the TPI sites has modified the understanding of humid tropics occupation by humans. The presence of ancient ruins, such as the Inca city *Machu Picchu*, used to be considered as evidence for developed societies. The absence of ruins in the Amazon forest was then considered indication for poorly developed societies, composed of small tribes living solely based on hunting, fishing and gathering activities. Nevertheless, the pre-Columbian indigenous groups living in the Amazonian forest subsisted on agriculture in addition to hunting, fishing and gathering activities [19], and their way of life generated the TPI areas of highly fertile soil, rich in plant nutrients [20]. The absence of ruins is now understood as a simple consequence of the sandy soil and gentle local climate.

In addition to their unusually high fertility over long periods of usage, TPI soils also present high resilience and stability, offering chemical recalcitrance even in the high temperature and humidity levels of the Amazonian climate [8]. The key aspect of the TPI sites is the presence of higher stable carbon content (approximately 70 times more than the surrounding soils) [15]. While the soil in the fertile sites exhibit similar textures and mineralogy to adjacent soils, the presence of a large amount of carbon in the TPIs is responsible for the stability and recalcitrance of the soil organic matter [8, 13, 21–23] and for a high soil cation exchange capacity, which has remained for thousands of years.

6.1.3 Understanding and Reproducing the Anthropogenic Model

The artificial reproduction of Amazonian anthropogenic TPI soils has been subject of many studies to improve soil quality [1, 3, 15, 24, 25]. However, charcoal is a disordered, nanostructured carbon that can be produced of various sorts. TPIs have twofold higher cation exchange capacity than adjacent soils with the same amount of carbon [13]. Studies in the field of carbon-related materials have developed different types of well-defined and disordered structures [26–28]. Here, we study

the structure of the carbon materials found in "Terra Preta de Índio" (TPI), which we refer to as TPI-carbons, to elucidate their morphological aspects and the surface science behind their functionality. We show that TPI-carbons exhibit a special nanostructure that might be considered to generate "Terra Preta Nova" (TPN).

Previous studies performed by ^{13}C Nuclear Magnetic Resonance (NMR) comparing the soil organic matter of TPIs with that found in adjacent soils have shown higher amounts of aromatic structures, and the process of burning (charring) of organic matter is responsible for the formation of such structures. [29, 30] Structural information about the TPI-carbons helps to reveal the origin of this material and to explain its superiority in terms of fertility. Recent studies [31] have shown that the dominant structure of the TPI-carbons is the more convenient to promote a balance that ensures, at the same time, the reactivity of the soil (necessary to an optimal flow of nutrients between the soil and plants) and differentiated stability that sustains the quality of these sites for periods of millennia [1, 31–33]. In this chapter we will discuss the structure of the TPI-carbons, starting with their morphology in the micrometer scale, in Sect. 6.2, followed by elemental (Sect. 6.3) and molecular (Sect. 6.4) analysis, and discussion on the nanometer scale morphology (Sect. 6.5) as the key aspects for the TPI functionality. Section 6.6 will summarize the chapter with a discussion about the production of the "Terra Preta Nova" (New Dark Earth).

6.2 Micro-scale Morphology and Adsorption

The "Terra Preta de Índio" samples discussed in the coming sections were collected near Manaus, Amazonas State, Brazil, from three sites: Serra Baixa—TPI_{SB}—(costa do Açutuba), Iranduba (Lat 3º 30′S, Long. 60º 20′W), Balbina—TPI_{BB}— Presidente Figueiredo (Lat 1º 54′S, Long. 59º 28′W) and Costa do Laranjal—TPI_{CL}— Manacapuru (Lat 3º 18′S, Long. 60º 33′W). The samples were extracted from surface layer (0–20 cm depth) using a Dutch auger. Charcoal samples are also analyzed, and they were produced at Instituto Nacional de Pesquisa da Amazonia (INPA), using Ingá wood (*Inga edulis* Mart.), a typical plant species of Amazon.

6.2.1 Electron Microscopy

Scanning Electron Microscopy (SEM) images of TPI-carbon grains exhibit a morphology in the micro-scale that is similar to that of charcoal, i.e. they generally show nonspherical particles, with random shapes and forms. The grains shape are consistent with the type of black carbon appearing naturally in nature, different from black carbons originating from synthetic laboratory methods, usually originated from petroleum, exhibiting more regular morphology [1, 34, 35]. Furthermore, both TPI-carbon grains and charcoal grains show stalk vessels, typical from plants.

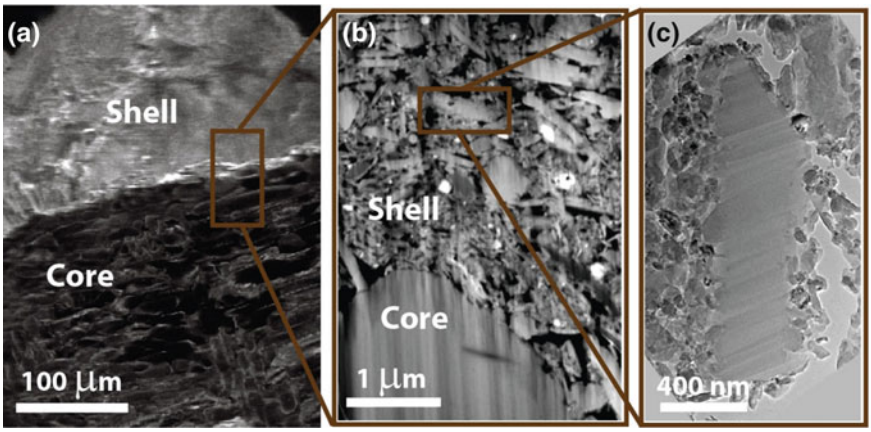

Fig. 6.1 **a** Scanning Electron Microscopy (SEM) image from a randomly selected TPI-carbon grain. The core and the surface shell are labeled. **b** Scanning transmission electron microscopy (STEM) image of a thin lamella showing the interface between the graphitic core and its porous nanostructured surface (shell). STEM images were acquired using a high-angle annular dark-field (HAADF) detector. **c** Transmission electron microscopy (TEM) image of single carbon nanoparticle in the porous nanostructured surface. The core-shell interface were cut using a focused ion beam (FIB) operated at dual beam platform using a Ga^+ ion source working at 30 keV accelerating energy for further TEM and STEM measurements [34]

Figure 6.1 shows electron microscopy images from a randomly collected TPI carbon grain at different zoom levels. The TPI-carbon grains exhibit a "fractal-like" structure, where a "compact core surrounded by porous shell" structure repeats itself when zooming in at different scales, from hundreds of micrometers down to nanometers.

Figure 6.1a shows a SEM image, where the frequently observed TPI-carbon morphology, with a compact carbon core presenting stalk vessels is surrounded by a porous shell. In a regular optical microscope, the core is graphite-black colored, while the shell presents a brighter contrast or a gray color.

The two topologically distinct zones are more evident in the zoomed scanning electron microscopy (STEM) image displayed in Fig. 6.1b. A thin cross-section was removed and welded onto a lift-out copper grid for such analysis. In the STEM analyses, one zone demonstrates continuous contrast, was microns in scale, composing the bulk grain core. The other zone located on the external part (surface) of the grain composes the shell. This region exhibits a nanometer-scale porous structure and is formed by aggregates of assembled 10–1000 nm particles. A TEM image of a typical nanometer carbon particle extracted from the external region of the TPI-carbon grain is shown in Fig. 6.1c. The particle surface is formed by smaller structures, which are several tens to hundreds of nanometers in length.

6.3 Elemental Analysis and Composition

6.3.1 Large Scale Analysis

The TPI's areas exhibit much larger concentration of nutrients, when compared with the surrounding soils. Table 6.1 shows quantitative large scale composition details of the TPIs collected from Serra Baixa (costa do Açutuba) and Costa do Laranjal [31]. The high fertility potential of the *Terra Preta de Índio* is, therefore, related to the large amounts of Ca, Mg, P, Cu, Zn and Mn presented in these soils (Table 6.1).

6.3.2 Microscopic Level Analysis

The chemical composition at the microscopic level has been obtained using EDX of hundreds of grains, revealing that besides carbon, silicon, and oxygen, common to all grains, elements like Al, Fe, Cl, Ca, Mg, P, K Ti, Na, Mn, Ti and Zr can be found. Both shape (see Sect. 6.2) and chemical composition are in agreement with the natural origin for the TPI grains, which is based on the aggregation of mineral and soil organic matter [1, 34, 35]. The main observations are (i) the core is composed mostly by carbon, but oxygen and calcium are spread over the whole structure; (ii) the other elements are located majorly in the grain shell.

Figure 6.2 shows an energy dispersive X-ray (EDX) microscopic mapping sequence for the carbon nanoparticle shown in Fig. 6.1c. The bright areas indicate the presence of the specific element indicated at the bottom of the figure. From these images we see that the core structure is made of carbon, as expected, but it is also rich in oxygen and calcium, both broadly diffused along the carbonaceous structure. Phosphorous, chlorine and nitrogen were also found in much smaller quantities in the carbon core. The particle contains abundant amounts of Fe, Al, Si, O and P on its border (see rightmost panel in Fig. 6.2), as well as smaller amounts of chlorine and nitrogen (not shown). These metal oxide-rich nanoparticles were visible on a scale of 10–100 nm. Overall, the elemental maps demonstrated the mechanism by which the porous surface structure helps to adsorb the complementary ingredients that contribute to the high fertility of "Terra Preta de Índio". The nutrient content, which is represented by the abundance of each element, vary largely among grains and among the sites of Amazonian Black Earth, which is in agreement with large scale chemical analysis [14].

More information about elemental analysis has been obtained by X-Ray Photoemission Spectroscopy (XPS). Similar to other productive soils [36], the TPI-carbon spectrum showed the presence of Ca, P, Fe, Al, Mg, Si and Na, as well as other elements with contents smaller than 1 % (K, Ti, Cr, Mn, Fe, Cu and Zn).

Table 6.1 Composition details for two "*Terra Preta de Índio*" sites [31]

Sites	pH H_2O	pH KCl	K (Cmolc kg^{-1})	Ca	Mg	Al	t	S	Fe (mg kg^{-1})	Zn	Mn	Cu	p	V (%)	M
Serra Baixa (Iranduba)	5.77	4.43	0.10	3.06	0.51	0.15	3.82	3.67	59.00	15.90	72.90	5.00	214.00	96.07	3.93
Costa Laranjal (Manacapuru)	5.52	5.23	0.17	9.38	1.30	0.05	10.89	10.84	63.20	292.30	843.30	8.00	285.76	99.54	0.46

t Effective cation exchange capacity (sum of K^+, Ca^{2+}, Mg and Al)
S Basis sum (K, Ca and Mg)
V Basis saturation percentage for effective cation exchange capacity
M Al saturation percentage for effective cation exchange capacity

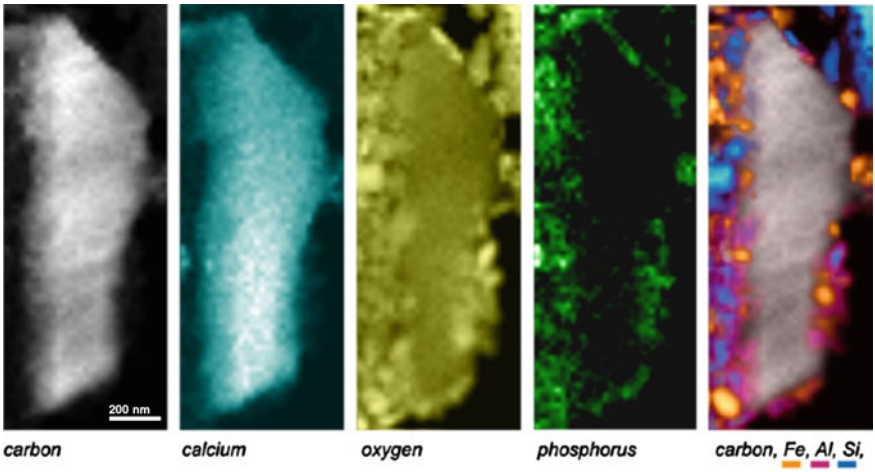

Fig. 6.2 Energy dispersive X-ray (EDX) of the nanostructured surface of a carbon nanoparticle to determine the local chemical composition; the various elements are labeled below each panel [31]

In particular, the presence of Ca and P in the TPI-carbons has been attributed to bone fragments and animal residue [37]. More details about XPS studies are found in the next section.

6.4 Molecular Analysis and Stability

6.4.1 Electron Energy Loss Spectroscopy

To probe the chemical state of both the core and the external regions of the TPI-carbon grains, spatially localized electron energy loss spectroscopy (EELS) have been performed, as shown in Fig. 6.3. [31, 38–40]. The energy loss regions investigated by EELS covered the carbon ionization K-edge around 285 eV. Chemical bonding maps were constructed from the energy loss near-edge fine structure (ELNES) of the carbon K-edge. This fine structure represents the transition from the Carbon 1s electronic core state to the unoccupied valence states (π^*) above the Fermi level. Well-defined energy peaks were observed in the EELS spectra and demonstrated the presence of ordered structures with signals representing carbon $1s \rightarrow \pi^*$ and $1s \rightarrow \sigma^*$ electronic transitions, which indicate sp^2 hybridization carbon structures. In the $1s \rightarrow \pi^*$ range, four well-defined main peaks were assigned: the aromatic-C peak at 285 eV, the phenol-C peak at 286.7 eV, the aromatic carbonyl peak at 287 eV and the carboxyl peak at 288.6 eV [35].

The relative peak intensities for the spectra shown in Fig. 6.3 indicate that the core is more graphitic than the surface, the external region of the grain demonstrating a deeper level of oxidation, which caused an increase in the abundance of

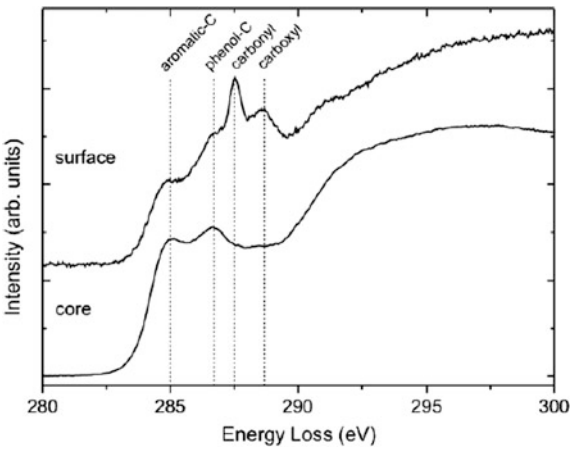

Fig. 6.3 Electron energy loss spectroscopy (EELS) analysis of the core (*bottom spectra*) and the surface (*top spectra*) of a TPI-carbon grain [31]

phenol, carbonyl and carboxylic groups. Spatially-resolved mapping of each chemical group have been performed [40], with spatially resolved measurement grid spaced by 15 nm from each other. The chemical maps show that the grain core has a tendency to be more aromatic (graphitic) than the grain surface (shell). In addition, the results clearly show that the aging induced oxidation is not homogeneous in the sample within the spatial resolution of 15 nm, revealing chemical domains that go from 20 nm^2 up to hundreds of nm^2. Higher oxidative-degradation levels indicated by the presence of carbonyl and carboxyl groups are more pronounced at the surface of the grain.

6.4.2 X-Ray Photoemission Spectroscopy

The calcium-oxygen-carbon interactions have been shown to be important for stabilization of the TPI carbons. To elucidate the calcium-oxygen-carbon at the molecular level in TPI, X-Ray Photoemission Spectroscopy (XPS) has been applied. Broad surveys of TPI-carbons and charcoal XPS spectra are shown in Fig. 6.4. It is evident that the TPI-carbons show a variety of elements in their composition, in contrast to charcoal, which shows essentially only C and O. From the XPS spectra, the core-electron binding energies of the elements found in TPI-carbons can be extracted. The binding energies are consistent with those obtained in silicates (P, Si), metallic oxides (Al, Ca, P, Fe) and metals adsorbed onto clayed materials (Mg, Ca, P) [36].

Detailed high resolution XPS analyses of the chemical groups in the TPI-carbons and charcoal containing carbon and oxygen show that the electron binding energies of carbon 1s (C 1s) are assigned to the functional groups usually found in soil organic carbon, i.e. sp^2 hybridized carbon (C sp^2), epoxide and hydroxyl, carbonyl,

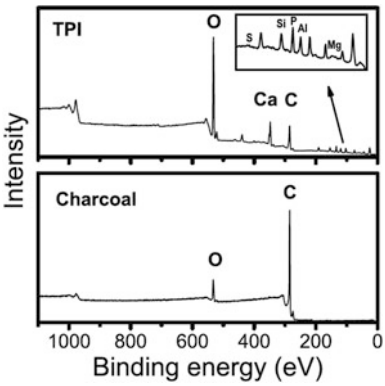

Fig. 6.4 Chemical characterization of the "Terra Preta de Índio" carbon nanostructures (TPI-carbons, *top*) and charcoal (*bottom*) using X-ray photoelectron spectroscopy (XPS). In contrast to charcoal, which essentially shows only carbon and oxygen, the TPI-carbons show a rich variety of elements, especially Ca, as indicated near the associated XPS peaks [40]

carboxylate and carbonate anions, in agreement with the EELS experiments. The electron binding energies of oxygen 1s (O 1s) are less resolved and are generally attributed to oxidized carbon and metal oxides [41–44].

The C 1s and O 1s XPS peaks of charcoal show only two major components [44], whereas the TPI-carbons show more complex spectra. Oxidized carbon species comprise 62 % of the TPI-carbons, while the remaining 38 % consists of sp^2 carbon [43]. For the charcoal sample, the ratio of sp^2 carbon to oxidized carbon species is much larger than in the TPI-carbons: 73 % sp^2 carbon to 27 % oxidized carbon species.

The highest fractions of carbon-oxygen bonding in TPI-carbons are from epoxide (C–O–C) and hydroxyl (C–OH) groups [42]. Carbonyl (C=O) and carboxylate groups (O–C=O–) are known to be located at the edges of carbon crystallites [41–44]. The O 1s peak appearing at 528.5 eV is attributed to oxygen-metal bonds, as it is also present in calcium oxide [45, 46].

The XPS peaks associated with calcium in the TPI-carbons show that Ca is present in at least five different configurations, corresponding to Ca $2p_{3/2}$ peaks at 345.6, 346.2, 346.8, 347.4 and 348.0 eV. These peaks are assigned to, respectively, calcium bound to oxygen, calcium ionically bound to hydroxyl [47], calcium bound to oxidized carbon, calcium-phosphate ($Ca_3(PO_4)_2$ and $CaHPO_4$) in mineral form [48–50], and Ca cations physisorbed on π-clouds of sp^2 carbon (Ca–C sp^2) [51, 52]. The component at 346.8 eV is the highest peak and is assigned to calcium bound to different forms of oxidized carbon atoms, such as carboxylate anions, carbonyl and epoxide groups [53–56].

6.4.3 Theoretical Calculations

Theoretical calculations based on density functional theory [57, 58] have been performed to understand the role of oxygen and calcium for the TPI-carbon stability. The presence of the above-mentioned chemical groups indicates the similarity, at the molecular level, between the TPI-carbons and graphene oxide [59–61]. It is well known that the most common oxide groups in graphene oxide are the epoxide and hydroxyl groups in the basal plane and the carboxyl group (and possibly some hydroxyl and carbonyl groups) at the crystallite edges [59–61]. This similarity was explored to construct simplified theoretical models for the TPI-carbons. A graphene nanocluster ($C_{54}H_{18}$ molecule) was then used as a host for various oxygen chemical groups and Ca^{2+}, making it possible to study their interactions.

The different chemical interactions between calcium and oxidized carbon were studied by considering six different configurations: (1) Ca^{2+} adsorption in the middle of the basal graphene plane, as shown in Fig. 6.5a, and the ideal situation of Ca^{2+} adsorption on pristine graphene; (2) Interaction between Ca^{2+} and epoxide groups, as shown in Fig. 6.5b, where the oxygen atom initially forms a "bridge" configuration between two neighboring carbon atoms, but the Ca^{2+} cation breaks one of the O-C bonds and binds strongly to the oxygen atom; (3) Interaction between Ca^{2+} and hydroxyl groups, as shown in Fig. 6.5c; (4) Interaction between Ca^{2+} and a carbonyl group at the edge, as shown in Fig. 6.5d; (5) Interaction between Ca^{2+} cations and carboxyl groups at the graphene nanocluster edge, as shown in Fig. 6.5e; (6) For the stable carboxylate anion, shown in Fig. 6.5f.

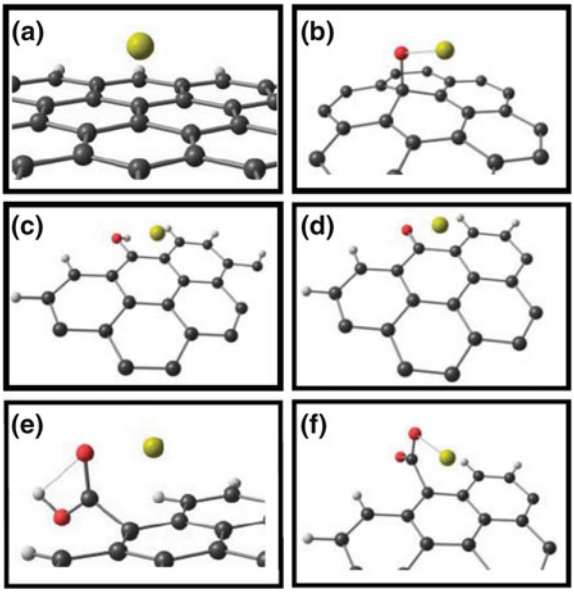

Fig. 6.5 Atomic geometries for the different structures considered in this work: **a** Ca–C sp^2; **b** Ca–epoxide; **c** Ca–hydroxyl (edge); **d** Ca–carbonyl; **e** Ca–carboxyl and **f** Ca–carboxylate. Carbon, hydrogen, oxygen and calcium atoms are represented by *black*, *white*, *red* and *yellow balls*, respectively [40]

The Ca^{2+} adsorption energies onto graphene oxide nanoclusters were calculated as the difference between the total energy of the Ca^{2+}–GO complex and the sum of total energies of GO and Ca^{2+}. The six models described above generate the following energy balance: (1) calculated adsorption energy of –121.4 kcal/mol, resulting from the interaction between the Ca^{2+} cation and the π-electrons of the basal plane; (2) adsorption energy of −146.6 kcal/mol. The enhancement in adsorption energy occurs because the Ca^{2+} cation is now in a more electronegative environment and interacts simultaneously with the epoxide group and the π-electrons, clearly enhancing the electrostatic interaction; (3) the Ca^{2+} binds very strongly to OH when the hydroxyl group is in the basal plane, removing it from the graphene oxide plane and then acting as a partial healer of the oxidized surface. Therefore, the hydroxyl–Ca^{2+}complex adsorbed on the basal plane is unstable. Most likely, a single Ca cation can remove two OH groups from the surface, thus forming $Ca(OH)_2$. However, if the hydroxyl group is placed at the crystallite edge, the Ca^{2+} cation does not remove the OH group. The Ca^{2+} cation actually binds to the OH group with an adsorption energy of −124.7 kcal/mol, as shown in Fig. 6.4c; (4) adsorption energy of −126.4 kcal/mol; (5) adsorption energy of −146.3 kcal/mol for the stable structure after geometrical optimization. Ca-carboxyl may not be observed experimentally because it is not stable with respect to the removal of one proton and transformation to Ca-carboxylate under typical soil pH conditions; (6) adsorption energy of −291.8 kcal/mol, showing the stronger electrostatic interaction with the Ca^{2+} cation. It is important to stress that, from a soil science perspective, it is well known that carboxylate anions play an important role in fertility [62]. The presence of acid groups in TPI surfaces makes the carbon surfaces more hydrophilic and decreases the pH of the point of zero charge. Structures containing more than one Ca^{2+} cation per oxygen chemical group were also investigated, but they were all unstable with respect to the desorption of the second cation. In other words, each of the studied chemical groups could hold only one Ca^{2+} cation and extra cations, if present, would move relatively freely through the TPI structure, which may have important implications for cation exchange considerations.

To gain insights about the importance of the primary grain sizes L_a, theoretical calculations were performed using the PM3 semiempirical quantum chemistry method [63], which has been shown to be suitable to study large graphene sheets and has been applied to study Li-ion batteries [64]. Several polycyclic hydrocarbon molecules with a general formula C_nH_m (n = 24–726; D_{6h} symmetry) were used as modes for graphene sheets. For this series, the diameter (L_a) ranged from 0.7 to 5.1 nm. Figure 6.6 plots the L_a dependence of the Ca(II)–graphene complex energy interaction obtained with quantum chemistry calculations [63, 64]. The stability of the Ca(II)–graphene complex decreases and the energy increases for sheets smaller than 3 nm in diameter, while for values larger than 3 nm, the interaction energies converge ($E_{int} \sim 190$ kcal/mol). However, for in-plane sizes $L_a > 8$ nm, the absorption and release of other elements into the graphitic structure is likely to be naturally reduced. Due to a decrease in the surface-to-volume ratio and interlayer distances [27], the nanographite particle tends to be an inert structure, similar to

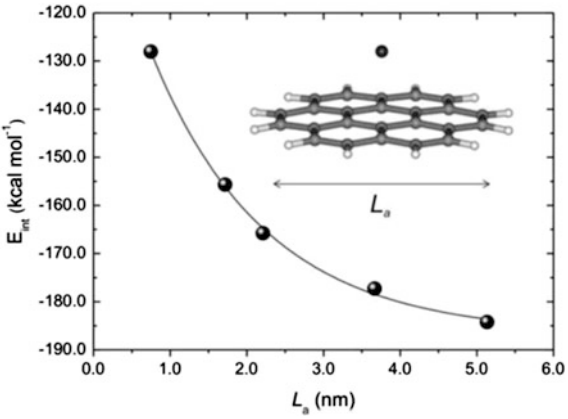

Fig. 6.6 Quantum chemistry analysis of the graphene–Ca(II) complex stability. The interaction energy is plotted as a function of the graphene plane diameter L_a. The calculations were performed using the PM3 semi-empirical quantum chemistry method [31]

bulk graphite. Therefore, the nanosized aspect of the carbon structure seems to be a key aspect in the TPI functionality.

6.5 Nano-scale Morphology as the Key for the TPI Functionality

In the previous section, Fig. 6.6 and related discussion show how the single-crystallite grain sizes can be important for the properties of the TPI carbons. Resonance Raman spectroscopy is a tool which provides structural information of carbon materials. In sp^2 layered carbons, Raman spectroscopy can be used to measure the nanocrystallite dimensions, which are defined by the in-plane crystallite size (L_a) [65–69]. The major spectroscopic signatures are given by two broad peaks, labeled G and D, at about 1580 cm^{-1} and 1350 cm^{-1}, respectively. These two features can be related to the presence of D_{6h}-symmetry carbon systems [65–72]. Raman spectroscopy measurements in TPI-carbons show a G-band frequency and a D to G integrated area ratio that are consistent with majorly sp^2 hybridized carbon nanocrystallites [31, 73].

6.5.1 Comparison Among Different Carbon Sources

Insight about the in-plane dimension L_a of the nanocrystalline structures composing these grains can be obtained from the analysis of the G peak full-width at half

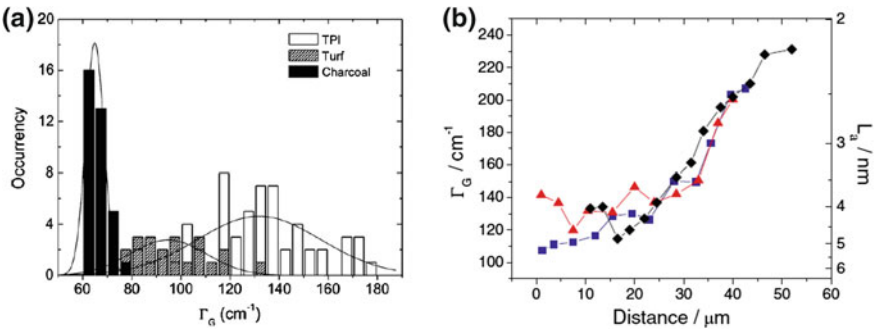

Fig. 6.7 **a** Statistical Raman spectroscopy analysis for crystallite dimensionality via the G band linewidth (Γ_G) [31]. **b** G band Raman spectra linewidth at different locations of an isolated TPI-carbon grain, moving from core to the surface. The Γ_G (or L_a) is plotted versus distance from the core to the surface for three different mapping procedures (*different symbols*). Measurements performed on a TPI-carbon grain found in the TPI samples. Data obtained with the 633 nm laser [73]

maximum (Γ_G) [65–72]. The importance of the Γ_G Raman analysis can be demonstrated by comparing results from the TPI-carbons with results from (i) turf (or peat), which is another highly productive black soil formed by the accumulation of partially decayed vegetative matter and (ii) different samples of charcoal from different vegetable sources that are intentionally produced for generation of TPN ("Terra Preta Nova" or New Black Earth). Figure 6.7a plots the measured distribution for Γ_G. The charcoal produced for TPN generation did not exhibit the expected carbon nanostructure because the Γ_G was too small (i.e. L_a was too large). Considering the $L_a \propto 1/\Gamma_G$ relation obtained for heat treated diamond-like carbons [68, 69, 73], the crystallite size distribution for a piece of charcoal was typically found between 8 and 12 nm, while the TPI-carbons were found in the L_a = 3–8 nm range.

6.5.2 *Analysis Within the TPI-Carbon Grain*

The considerable spread in Γ_G (or L_a) for the measured TPI-carbons were shown to be due to different structures spread within the core and the surface of the grains. The mapping of the Raman spectra from core to grain surface is capable to reveal changes in the crystallite structure, as shown in Fig. 6.7b. A TPI carbon grain was sectioned, thus making it possible to measure specific points from the interior (core) to the exterior (surface) region of the TPI-carbon grain. Raman spectra were taken from the core to the surface of the TPI-carbon grain, and comparing the spectra, there is an evolution of overlap between the D and G peaks, related to an increase in Γ_G. Using the relation $L_a = 496/(\Gamma_G - 15)$ that was developed for the TPI carbons [73], the average crystallite size in the center of the carbon structure is L_a (core) = 5.3 nm, while at the surface L_a (surface) = 2.6 nm. Figure 6.7b corresponds to the relation between Γ_G (and L_a) and the distance from the core to the surface for

three mapping procedures. A gradual increase in Γ_G (i.e. decrease in L_a) is observed when the laser focus is moved from the core to the surface. These results indicate that the disordering (oxidation) process was more effective at the surface, in agreement with previously published analysis [31, 38]. The reactivity is provided by smaller crystallite sizes in the periphery, whereas the stability of this material is related to larger crystallite sizes dominant in the core. This aspect seems to be important for both the stability of the carbon structure and for improving the cation absorption and release in the graphitic structure.

Recently, the depth dependence of the properties from a soil collected from the Balbina site, in Presidente Figueiredo, Amazonas State, Brazil, was studied [74]. The black carbon structure and the soil composition were investigated, with special emphasis on the poorly studied microbiological composition. The comparative analysis between the properties from newer (shallower) and older (deeper) soil strata indicates that, while soil composition exhibits depth dependence, the pyrogenic black carbon structure does not. This finding suggests that this model material should be reproducible by repeating the pyrolysis conditions utilized in their production.

6.6 Summary and Perspectives

Reproducing the anthropogenic model "Terra Preta de Índio" (TPI) is expected to have big impact in soil amelioration and carbon storage, especially for life in the humid tropics. To date, scientists have been unable to completely reproduce the beneficial properties of TPI. It is believed that the special black carbon (or biochar) present in the TPI is responsible for water retention, high levels of cation exchange, resiliency and other beneficial properties of the TPI. However, fresh charcoal must first be "changed" before it can function as a biotope.

By applying the knowledge in the field of carbon materials science to address a complex problem related to soil science, it seems consistent that the millenary carbon phase in TPI started as a charcoal-like structure, as believed by soil scientists. This type of structure is stable and does not leach. In sequence, the environment action over thousands of years has degraded the surface and, as a result, the in-plane crystallite size (L_a) was diminished towards maximum catalytic functionality, allowing the formation of the unusually highly fertile soils.

It is clear that the external region of the grains contributes significantly to the high cation exchange capacity of the soil, while the graphitic core structures act as a sink for cations, such as Ca, and also as a reservoir of graphitic structure for millennia of degradation. This type of degradation increases the cation exchange capacity of the soil, its ability to retain nutrients and its fertility. The large amount of organic residues from animal and vegetative origin provides macronutrients and micronutrients retained by the carbon nanoparticles with primary particles in the 2–8 nm scale. Our results clearly demonstrate the strong chemical stability of calcium in TPI, with adsorption energies as high as −291.8 kcal/mol per calcium

cation. The energy values are well within the chemisorption regime and arise from strong electrostatic (ionic) interactions. The agreement between the calculated and experimental core-electron binding energies confirms the validity of our molecular models.

The critical value of about 2–8 nm for graphitic nanocrystallites found for the TPI-carbons implies an active structure that reconciles functionality with stability [31, 73]. Slow partial oxidation and/or aggregation of organic molecules present in soil organic matter during thousands of years produce the oxidized carbon at the surface, which is not fully homogeneous at the nanoscale, where different carbon-oxygen functional groups, such as phenol-C, carbonyl-C and carboxyl-C are observed to dominate the spectra at different areas, in the range of tens of nanometers. When compared to present-day fresh charcoal, the spectral characteristics of TPI-carbon grain cores are similar, presenting low level of oxidation and low amounts of carbonyl and carboxyl groups.

The TPI-carbon serves as a colloidal mineral that regulates the retention and delivery of nutrients from the soil solution to the root plant system, and at the same time it is responsible for resiliency of the soil. The inhomogeneous distribution of the different functional groups at the tens of nanometer scale indicates that a non-simultaneous oxidation and calcium adsorption favors different carbon-oxygen-calcium functional groups functionality.

Our analysis is certainly limited when considering the complexity of the problem, but it is clear that the structural aspects of the TPI-carbons are different from those of recently produced charcoal, which is consistent with the fact that the simple addition of charcoal as a soil amendment does not generate an efficient TPN. For TPN generation, agronomists may have to consider the use of amorphous carbon nanostructures with the proprieties reported here. The action of biological agents, such as fungi found in the soil has to be addressed as well, to understand further details in the TPI functionality [74].

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Chapter 7
Nanotoxicology of Carbon-Based Nanomaterials

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Abstract Carbon-based nanomaterials are promising building blocks in a myriad of technological applications ranging from microprocessors to drug delivery. These expanding applications provided by the nanotechnological context will possibly result in a future exposure of human beings and environments to these carbon-based nanomaterials. In this new scenario, the perspectives of using carbon-based nanomaterials must be tightly linked with the development of a detailed understanding of their toxicity. The potential risks related to the production and application of carbon-related materials have been assessed in an increasing number of nanotoxicological studies on these nanostructures performed in the past fifteen years. Scientific community has been observing bioeffects manifested from the interactions between carbon nanostructures and biological entities organized in different levels of complexity, ranging from biomolecules to living organisms, but the large amount of data generated in these studies are still not integrated. In view of the necessity of integrating these results in the context of carbon nanostructures nanotoxicity, this chapter discusses significant toxicological assessments in the sense of enlightening possible biological effects manifested from the interaction of carbon-based nanomaterials and biological entities organized in different levels of complexity. The chapter covers results of in vitro and in vivo toxicological assessments of carbon nanostructures that show conditions in which these nanomaterials can be degraded by biological systems. Furthermore, we have introduced

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discussion not only on the different aspects of toxicity of these nanomaterials but also on the methodology used in each scenario. Finally, it is presented a properly correlation between the biological phenomena and the structural and morphological differences intrinsically present in carbon nanostructures that result from synthetic and processing variations.

7.1 Carbon Nanomaterials

Carbon is one of the most abundant elements in nature forming different allotropes, i.e., carbon atoms linked together in different ways, which results in a range of chemical structures with different properties and therefore numerous materials. Thus, carbon materials are used when there is the need for extremely hard materials (diamond), strength (carbon composites), porosity (activated carbon), electric conduction (graphite), and insulation (glassy carbon). Furthermore, carbon-based materials are useful for applications in extreme conditions, such as high temperatures.

A brief history of carbon materials demonstrate the importance of these materials in the technological advancement of our society, from prehistoric times with rock paintings, development of gunpowder and writing; through the 60's with the development of the high-strength and flexible carbon fibers and hard vitreous-glassy carbon; the 70's with the development of heart valves and prosthetic carbon-based materials of excellent biocompatibility; and finally the 80's when carbon materials (isotropic graphite blocks of high density) were able to improve the capacity of high temperature reactors and the electrodes for electric discharge. Carbon materials have gained more prominence with the discovery of nanostructured carbons; fullerenes in mid 80's [1], carbon nanotubes in early 90's [2] and graphene in the past decade [3]. These systems have been responsible for moving forward the research on nanoscience and nanotechnology because their outstanding properties make them ideal model systems. The fast development of carbon nanoscience also benefits from the institutionalization of nanotechnology as science policy in the U.S. in 2000, which spread out around the globe as a development platform in several countries. As the industry realized that nanotechnology is a platform for future technologies, funding research in this area as well as in nanoscience has been growing progressively. In particular, big companies are testing carbon nanomaterials for electronics based on ultra-small and ultra-fast devices that could one day replace the silicon chip [4].

Graphene is the name given to a sheet (the thinnest layer possible in nature) of carbon atoms arranged into two-dimensional graphitic structure with a honeycomb-like crystalline structure, which is just one layer of graphite. This layer can be considered the "mother structure" for understanding the properties and conceptually building the other well-known sp^2-based nanocarbons such as fullerenes; carbon nanotubes (CNTs) and carbon nanoribbons [5]. All of these nanocarbons materials are stars in material sciences not only because their science

is very rich but also because they remarkable properties are generating many proofs of concept for applications in many technological areas. Graphene is simultaneously the stronger and thinner material ever known, and it is also an excellent conductor of heat and electricity [4]. Several studies, are evaluating from their basic properties to potential applications in electronics devices [6–12]. Carbon nanotubes have been considered as revolutionary materials in various areas such as electronics, polymer industry, sensors, catalysis, drug delivery and agronomics [13–20].

The thin layers of carbon atoms arranged in the honeycomb structure, sp^2 hybridization, and the presence of π electrons are the basis for the exceptional properties of graphene and CNTs. C–C bonds between two sp^2 carbons is the strongest chemical bond observed in nature and it can render a material with high strength and low weight. For instance, an hypothetical layer of 1.0 m^2 of graphene would have a mass of 0.77 mg and would support the a mass of 4.0 kg [4]. This network of symmetrically distributed carbon is also responsible for a good electrical conductivity and lower heat production, reason why graphene and CNTs are being considered as key materials for electronics. Furthermore, their potential for a industrial-technical breakthrough is also based on the fact that minor variations in the carbon structure (e.g. doping) can create a plurality of new properties [4]. CNTs have also a high potential for application in plasma and liquid crystal displays due to their ability to emit electrons from their tips to excite phosphor screens [21, 22]. Due to the ability of fullerene to act as an effective antioxidant, producing singlet oxygen species that act as scavengers radicals, this carbon material is considered to be a promising material in nanomedicine for the treatment of cancer [23]. However, associated with the potential of fullerene, its biotransformation and its derivatives were studied in parallel once C_{60} may have a long biological residence time [24]. Other promising perspectives of using carbon nanomaterials have arisen when they are used conjugated with resins or polymers impeding the propagation of cracks and crevices in composites, helping to dissipate heat and charge.

Along with the scientific and technological development related to carbon nanomaterials, and considering all the perspectives of applications abovementioned, it becomes clear and relevant the role that nanotoxicology can play in the sense of ensuring a safe use of commercial products containing these nanostructures. Furthermore, nanotoxicology is also advancing along with nanobiotechnology in order to introduce pro-active approaches for the evaluation of carbon nanomaterials safety issues whereby many applications in medicine have been proposed.

7.2 Toxicology Versus Nanotoxicology

Classical definitions of toxicology are related with the understanding of the effects manifested when chemical, physical or biological agents interact with living organisms and environment. Following this concept, toxicological approaches have been used since a long time ago: there are records that the father of Chinese medicine Shen Nung (mid-3000 BC) tasted 365 herbs and dyed from a toxic

overdose; the ancient Egyptian record named The Ebers papyrus (from approximately 1500 BC) contain information of toxic substances including hemlock; Roman philosopher and medical writer Aulus Cornelius Celsus recognized the antiseptic effect of vinegar; alchemist Paracelsus (1493–1541) pioneered the formal study of poisons; English surgeon Percivall Pott firstly recognized that occupational exposure of chimney sweeps could lead to cancer; and Alice Hamilton related the effects of lead and the development of rubber industry to the workers health [25].

Toxicological methods based firstly on the dose-response principle were introduced by Paracelsus: "All substances are toxic; there is none, which is not a poison. The right dose differentiates a poison from a remedy." [26] However, other parameters further discovered and that were not introduced by Paracelsus contributed for shedding light on the mechanisms behind the toxicological response, which often operates with a non-linear dose-response relationship. Other parameters introduced such as concentration and time exposure are also key points to determine the safety and hazard thresholds of a specific stressor agent. [27].

Recently, the development of nanotechnology allowed the creation of systems with controlled properties and designed for specific applications, many questions and concerns on the possible behavior and interactions manifested when nanostructures are in contact with biological entities arised. In this new panorama, nanotoxicology emerged as the "science of engineered nanodevices and nanostructures that deals with their effects in living organisms" [28]. Whereas classical toxicology has established robust protocols to deal with the effects of molecules and bulk materials acting on living systems, nanotoxicology is now introducing and developing standard methods for assessing materials toxicity in nanoscale, whose entity behavior and mechanisms of action are still not clear. Fundamentally, it is well know today that a material bulk when is fragmented into smaller pieces there is the manifestation of singular physical and chemical properties which can impact either positively or negatively their interactions with living beings and environment [29].

Undoubtedly, the importance given to the possible adverse effects of nanomaterials is based on the precaution principles due to previous disastrous experiences the humanity have faced long her recent history. With the advent of the industrial revolution in the nineteenth century, Asbestos became widely used without proactive studies and their toxicological potential was assessed only after a large-scale industrial production and reflected on harmful consequences to human health, such as asbestosis (chronic lung disease of occupational origin), lung cancer, gastrointestinal inflammation, and others. According to the World Health Organization (WHO), only in 2004 there were 107,000 deaths related to occupational exposure to asbestos [30].

Exposure to aerosols is one of the current examples of biological interaction of nanoparticles that are now being revisited in the context of nanotoxicology. Künzli et al. [31, 32] observed that air pollution from the combustion process appears to negatively affect mortality of individuals. These particles dispersed in the air can be a potential public health problem. Data considering carbon nanoparticles as the dispersed particles in the air are still scarce, mainly because the nanotoxicological studies are delayed compared to the nanotechnology development. However, some

studies conceived in a different context are helping in the evaluation of possible epidemiological trends for engineered nanoparticles in the long term, such as those that considered ultrafine particles (<100 nm) in air pollution [33].

In the specific case of carbon nanomaterials, nanotoxicology has been selecting parameters (such as dose, mass, number of particles, size, volume, surface chemistry and aggregation) that must be take into account when one is evaluating the toxicology of these structures. In this chapter we will emphasize these parameters and evaluate how they influence the biological effects manifested from their interaction with living systems and environment. Furthermore, the differences between scientific approaches used in classical toxicology and emerging nanotoxicology are highlighted as well.

7.3 Nanotoxicological Parameters Used for Carbon Nanomaterials

A meaningful parameter to be considered in nanotoxicology that has not been widely used in the toxicological evaluation of molecules is the surface area. When one considers that the surface atoms or molecules play a dominant role in the nanomaterial properties, chemical interactions manifested for a specific nanomaterial is possibly ruled by surface as well. Thus, by considering a mass of particles with identical chemical composition and crystalline structure, the main variable do differentiate these systems is the size of the particle and consequently the surface area. By increasing the surface area the material tends to increase its reactivity [34, 35], and when they are evaluated in toxicological assays it is commonly observed a larger toxicity effect for nanoparticles as compared with the bulk counterpart. Therefore, it is important to note that the organisms will present a different biological response when they are exposed to the same dose, in mass or molar units, of a nanostructured agent or other microstructured agent as compared with bulk material. In this way, adjustments in dose and exposure time are required depending on the selected agent to be tested. The increase of the surface area can also reflect on secondary biological effects such as antioxidant activity, penetration of cellular barriers, among others [28].

Along with the surface characteristics of the nanomaterial (e.g. surface area), chemical composition is obviously included as a main factor that influence on the toxicologic response [36]. For the specific case of nanocarbon materials, fullerene (C_{60}), carbon nanotubes (CNTs) and graphene are all carbon allotropes with similar chemical composition but different crystalline structure, which reflects in different responses to cellular uptake, intracellular localization and ability to catalyze oxidative stress, and others [37].

In general, there is no consensus in the literature regarding the concentration of a specific nanoparticle necessary to cause a toxic reaction, but it is well known that the nanoparticle aggregation state is strongly correlated with the discrepancies

between the results obtained by different groups. For a nanoparticle aggregate, the surface charge, chemical composition, and nanoparticle size are critical in the formation of nanoclusters, which are very unstable and this issue represent a complex parameter to be dealt by nanotoxicology. For instance, the variation of the nanoparticle morphology (i.e. size and shape) has a direct impact on the macrophages behavior because they can more effectively uptake particles with larger dimensions (100–200 nm) [28, 38] compared to smaller ones. Thus, in some cases nanoparticles in high concentration tend to aggregate [36, 39] and, consequently, reduce its toxicity compared to when they are dispersed in low concentrations [38]. In the latter case, nanoparticles appear to escape more easily from the defense mechanisms. Therefore, the complete and detailed characterization of the nanomaterial using several physico-chemical techniques is the first step towards the definition of biological effects studied under the context of nanotoxicology.

For nanotoxicology, the aspect ratio (length/diameter) of the nanomaterial is also directly related with the toxicity of the nanoparticle, as previously observed for fibers. Lippmann has reported that the higher the aspect ratio the more toxic is the particle [40]. In this sense, different lengths of asbestos are responsible for different biological reactions. Particles of asbestos (thickness around 150 nm) larger than 10 microns accumulated in the lungs tend to induce lung cancer while particles longer than 5 microns tend to induce mesothelioma; and those longer than 2 microns lead to asbestosis [40]. For carbon nanomaterials, the aspect ratio has been one of the main parameters evaluated in nanotoxicological approaches, mainly applied for CNTs when compared to carbon fibers and carbon black (Fig. 7.1) [41]. In fact, a comparison between CNTs of very different lengths indicated that their toxicological behavior to the mesothelial lining of the body cavity of mice has a length dependency similar to that reported for asbestos [42]. The limiting factor for this reaction

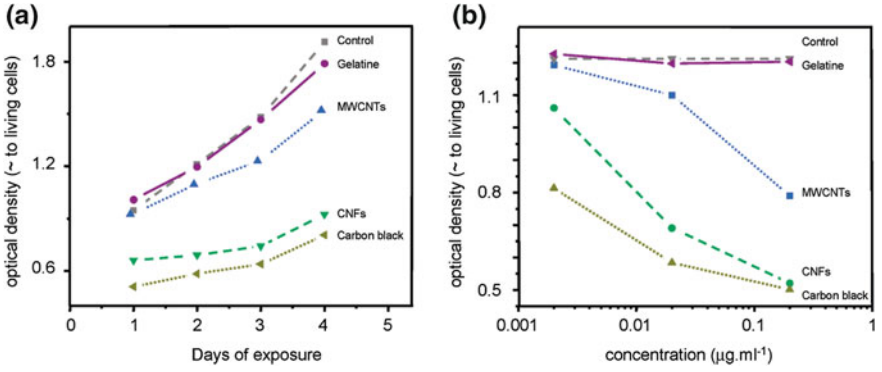

Fig. 7.1 **a** Representative growth curve for H596 cells grown in normal medium (control), medium containing gelatin, or 0.02 µg mL^{-1} of carbon nanomaterials (CBNs). MWCNTs, carbon nanofibers (CNFs), and flake-like shaped carbon nanoparticles (carbon black) are dispersed in the same gelatin-containing medium. **b** Dose-dependent toxicity of H596 cells exposed to CBNs for 2 days. Reprinted with permission from [41]. Copyright 2006 American Chemical Society

in the lungs is that alveolar macrophages have an average diameter of 14–21 μm [43]. Thus, particles larger than 20 microns cannot be effectively eliminated from the respiratory tract [44] where their biopersistence may lead to the development of numerous diseases as mentioned above. Other studies also suggested that CNTs can impose a potential toxicity to the body, primarily due to their morphological similarity to asbestos [45, 46]. Unusual inflammatory and fibrogenic pulmonary responses to single walled carbon nanotubes in mice have also been reported in the last decade [47–49].

The time (i.e. exposure time) that nanoparticles interact with the biological medium is a fundamental parameter for evaluating toxicity. The longer the time of contact with a living organism, the greater the probability that an adverse biological response could manifest. Certainly this depends on intrinsic properties of each nanomaterial such as solubility, dispersibility and hardness (i.e. breaking of nanoparticles generates shorter ones that can be phagocyted), which will reflect on its mean residence time in the organism [44]. For instance, soluble asbestos fibers are degraded in the body for months, while insoluble fibers remain in the lung indefinitely, leading to the release of cytokines, increasing the levels of reactive oxygen species and other chemical mediators. Some carbon nanotubes present a very slow elimination or in fact remain indefinitely in the lungs after administration through the respiratory tract. In this particular case, more than 80 % of CNTs were found in the lungs of mice after 60 days of exposure [45].

Several nanobiotechnological applications demand surface modifications of nanomaterials by chemical functionalizations, with the aim of improving chemical processability and functionality. This is the case of graphene and carbon nanotubes, which can improve their dispersibility in water or organic solvents through these processes [50]. The most used functionalization in sp^2 carbon nanostructures is the oxidation. Through this process, the chemical bonds between sp^2-carbons, present in the walls of carbon nanotubes and graphene are broken with the formation of oxygenated groups such as esters, ethers, alcohols, carboxylic acids and anhydrides. When oxidized, these materials are called graphene oxide (GO) and oxidized carbon nanotubes (o-CNTs). Among the oxygenated groups generated at the surface, carboxylic acids groups can provide a dramatic increase in chemical processability for graphene oxide and oxidized carbon nanotubes, because the carboxylated nanostructures can be easily dispersible in water and their chemistry is rich enough for performing a myriad of chemical reactions. These changes in the chemical structure will depend on the parameters used during the oxidation process. By controlling the process, it is possible to generate a small amount of oxygenated groups enough for them to be dispersible in water, whereas most of the graphitic structure is still preserved. However, these surface modifications can largely alter the surface characteristics of the nanoparticles and thus modify its toxicology. In some situations, harmful nanostructures can become non-toxic whereas nanostructures with little toxicity can become highly toxic as a function of chemical surface functionalizations. For instance, CdSe quantum dots can be rendered non-toxic when appropriately coated with mercaptoacetic acid [51]. CNTs in particular have been applied in several biological contexts after undergoing to covalent

and non-covalent functionalizations, specially oxidation reactions, in order not only to provide new physical-chemical properties, but also to change the toxicity [52].

The myriad of characteristics of carbon nanomaterials such as morphology, size, and surface chemical functionalization directly influence on the toxicology of these materials. CNTs may present as single-walled, multi-walled, with closed and open ends, with diameters ranging from 0.4 to 100 nm, length ranging from nanometers to centimeters, and with different states of aggregation due to their hydrophobicity [46, 49, 53]. Surface treatments (e.g. oxidation) must be also considered, and may lead to the formation of hydrophilic groups such as carbonyl and hydroxyl, largely localized at the edges and defects, contributing for the increase of dispersibility in water. What can be observed in the literature is that some studies report that CNTs have high toxicological potential in different human cells, but the dose is still very controversial mainly due to the intrinsic variation of morphology and chemical structure between samples produced around the globe. For instance, Bottini et al. [54] observed that 400 μg mL^{-1} of multi-walled CNTs (MWCNTs) were able to induce the death of human T cells. Jia et al. pointed out that 3.06 μg cm^{-2} of MWCNTs can cause the apoptosis of alveolar macrophages [55]. Cherukuri et al. evaluated doses of single-walled CNTs (SWCNTs) below 3.8 μg mL^{-1} and observed no cytotoxicity against phagocytic cells, although it was observed that cells produce proteins in an attempt to coat and aggregate SWCNTs [56]. Additionally, SWCNTs produced significant pulmonary toxicity when compared with amorphous carbon black-spherical particles [48, 54, 57] and Pharyngeal introduction of SWCNTs induced acute inflammation in rats with early and progressive granulomas and fibrosis [48, 57].

To understand the variety of in vitro results reported so far for carbon nanomaterials and to relate these bio-effects to the nanostructure characteristic, the biomolecule-nanostructure interaction is one the first phenomena to be addressed. When in contact with functional biomolecules, nanoparticles induce the manifestation of complex interactions which are established with components of the biological medium. These interactions are manifested because both entities (i.e. nanoparticle and biomolecule) have the same order of size and possess suitable sites for interaction. Furthermore, this phenomenon does not obey linear relationships among the variables involved. In this scenario, it must be considered that these interactions occurring at the "bio-nano" interface may alter completely physical and chemical properties of the nano-object, thus altering the toxicity as well. Analogously, one can consider the effect that surfactants can induce on nanoparticles: they are able to alter optical, electrical properties, and the reactivity of nanoparticles [28, 58, 59], thus impacting key features related to the toxicity of these materials. For instance, it is well known that nanoparticle surfaces containing groups O_2, O_3, and some metals induce an inflammatory process [60, 61] whereas fullerene (C_{60}) molecules induce lipid oxidation in human tumor cells (skin and liver) [61]. All these biological effects induced by nanoparticles in a living organism will be mediated by the biomolecules (e.g. proteins) which are chemically interacting with the nanomaterial. The scenario in which nanoparticles interact with living cells and organs through their bare surface (without a biomolecular coating) is unrealistic.

Consequently, a nanoparticle when in contact with a biological environment containing biomolecules (mainly proteins) will possess a molecular layer on its surface named *protein corona*. The protein corona formed on the nanoparticle is then an extrinsic feature of the nanoparticle which is strongly related with its characteristics as well as with the biological medium in which it is interacting. The complexity of this interaction arises from the ability of the nanoparticle to bind selectively (non-covalently) with biological entities depending on its size, chemical composition, morphology, topography, and functionalizations. Thus, it is necessary to understand precisely not only the nanomaterial characteristic but also the particular interactions that this entity will develop in a determined biological context of application, because at the end point which interacts with the cells is not the bare nanoparticle but the hybrid consisting of the nanoparticle plus protein corona. Currently this interaction mechanism is not fully elucidated due to the huge amount of variables involved [62, 63].

Along the past fifteen years, fundamental knowledge was generated and several papers reported biological reactions to carbon nanostructures with different morphologies, chemical structures, and functionalizations. However, these recent studies on the formation of protein coronas have presented a new paradigm for the field of nanobiotechnology and nanotoxicology, which demonstrated that the possible effects of interactions occurring at this interface involve not only the chemical characteristics of the surface of the nanomaterial, but also the existence of a new entity formed by the nanoparticle itself plus the coating formed with biomolecules over the nanoparticle, which will depend on the context or administration route (Fig. 7.2) [63–65]. In the particular case of blood plasma, the surface microchemical environment of the nanoparticle is the result of the biomolecular coating formed by supramolecular interactions (e.g. electrostatic and hydrogen bonds) and composed primarily of proteins, which make the adsorption energetically favorable, since this new nanometric entity is very stable in the long term. In this new realistic approach which defines in fact what the living system will "see"

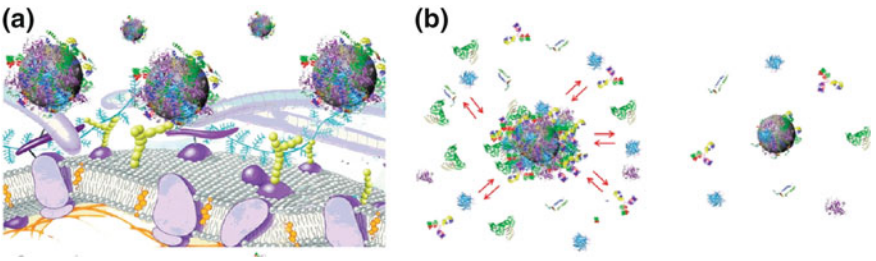

Fig. 7.2 **a** Cartoon representation of the possible exchange/interaction scenarios at the bionanointerface at the cellular level. **b** Schematic drawing of the structure of NP-protein complexes in plasma: the "core" nanoparticle is surrounded by the protein corona composed of an outer weakly interacting layer of protein (*left, full red arrows*) rapidly exchanging with a collection of free proteins and a "hard" slowly exchanging corona of proteins (*right*). Reprinted with permission from [65]. Copyright 2010 American Chemical Society

and “feel”, cytotoxicity, for instance, will manifest due to the interactions of cells with such biomolecular coating on the nanoparticle and not simply due to the chemical nature of the surface of the nanomaterial. The importance of these results exceeded the domain of nanotoxicology once these phenomena must be considered in all nanobiotechnological applications of nanostructures. Furthermore, it is now clear that nanobiotechnology must use methods and scientific approaches based on Complexity, once it is dealing with the specificity and variety involved in the weak chemical interactions of living matter. Another important observation was of the inherent dynamism related to the bio-nano interactions that is characteristic to any type of nanostructure in the context of biological applications.

With these experimental findings involving the formation of biomolecular coatings on nanostructures, one have to consider that the effect expected from a specific chemical functionalization on the surface of the nanoparticles can be modified in certain biological media by the presence of bio-nano interactions. In order to illustrate this point, we can consider the hemolytic effect, parameter widely used to evaluate the biocompatibility of nanoparticles when intravenous administration is aimed. In the particular case of silica nanoparticles, as the toxic effect of the silanol (Si-OH) groups to the red blood cells of mammals is well known [66, 67], a prerequisite for the intravenous administration of these nanomaterials is the reduction of their toxicity. Thus, the influence of the chemical characteristics of the surface of silica nanoparticles on the hemolytic effect were studied as well as other types of SiO_2 nanostructures were also tested intending to find the best combinations of morphology, pore structure and surface chemical functionalization [68, 69]. However, in all hemolytic studies presented it was not considered the presence of biomolecules from blood plasma, once hemolytic tests were made in phosphate buffer solution (PBS). In a more realistic model, the dispersion of silica nanoparticles in blood plasma will likely lead to the formation of interactions between the surface of nanoparticles and biomolecules. Therefore, biomolecular coatings can be generated and the effects of toxicity evaluated can be completely modified due to the existence of an entirely different surface microchemical environment from that of bare nanoparticles, being now defined by the chemical characteristics of the adsorbed biomolecules. In the specific case of silica nanoparticles, the hemolytic effect is suppressed by the presence of proteins present in blood plasma after their adsorption on the nanoparticle [70]. Knowing these possibilities, the surface functionalizations on nanoparticles should maintain their function even in the face of this interactional competition. Therefore, it is important firstly to think about the interactional compatibility depending on the size order of the chemical entities present in the bio-nano interface. For instance, one can consider that a propylamine group covalently attached to a nanoparticle surface (functionalization that was used in a huge amount of works) can have their chemical characteristics canceled by the biomolecular deposition caused by the adsorption of proteins with a larger chemical structure.

Including all abovementioned parameters influencing on the biological effect, besides the well-done characterization of nanomaterials, nanotoxicology must also deal with different kind of expositions that the biological entity may face. In this

context, nanoparticles can be dealt as part of a bulk materials (immobilized) or as free particles in a particular medium [71]. Transitions between these two states are also of extreme relevance to nanotoxicology. Furthermore, in the case of immobilization, nanoparticles can also be differentiated with respect to their location: within the volume or on the surface of a solid material. Liquid or gas media of dispersion must also be well differentiated in this case. For the particular case of a toxic nanomaterial dispersed in a gas medium, there must be considered a higher risk associated to the larger spreading capacity, which may lead to a wide range dispersion of nanoparticles with little control.

Therefore, classical toxicology protocols make use of well-established methodologies correlating dose-effect approaches. Nanotoxicology however, which is being currently developed, appears to be a more complex science in which the dose does not follow linear relationships with the biological responses in several cases, thus being relevant to assess the effect of low doses used in the bionanotechnological context instead of high doses used in classical toxicology, once the latter is easy to be detected. In contrast, a chronic low-dose exposure to nanomaterials can become a health issue once nanomaterials could lead to diseases from chronic and long-term exposures. Thus, it becomes essential to determine the number of particles that reach each cell [72, 73]. As observed, protocols and bioassays for nanoparticles are still under development once they have a set of characteristics such as size, shape, surface charge, surface coating, dispersion features, agglomeration aspects, concentration and the medium in which they are dispersed. This set of characteristics can also be modified along the time of exposure. Furthermore, this complexity can increase when considered different biological media, such as those present in lungs, intestine and skin. In addition, non-dispersible nanoparticles administered via a particular way can be translocated in the living organism through biological barriers which can modify the nanoparticle features along the pathway in a very specific way [74].

Nanotoxicology is thus a new science which is currently establishing standard protocols and characterization methods able to deal with the nanostructure peculiarities that have been gradually revealed by the scientific community. At first glance, classical protocols are being used as the starting point and basis for the advancement of the area. Well-established toxicological mechanisms (e.g. generation of reactive oxygen species, protein misfolding, membrane disruption) have also been identified and successfully used for understanding interactions of nanomaterials in several biological contexts [75]. However, some basic challenges still need to be overcome, such as determining the actual dose of nanoparticles used in experiments. Furthermore, the nature and consequences of the bio-nano interactions must also be studied for living systems organized in different levels of complexities. For this particular issue, in vivo studies are gradually enlightening issues which are still unclear from in vitro results. Then, nanotoxicology is progressively assessing the safety and trying to determine undesirable effects of nanomaterials to animals, human beings and environment.

7.4 Interactions of Carbon Nanomaterials with Biomolecules, Microorganisms and Cells

7.4.1 Carbon Nanotubes and Enzymes

Scientific community is currently striving to evaluate the toxicity of carbon nanomaterials, considering that differences among CNTs, graphene and fullerene reflect on different bio-effects in animal, humans and also on the environment [76]. In the specific case of carbon nanotubes, although their great potential for biomedical applications, many toxic effects have been observed from their interaction with biological systems (e.g. oxidative stress, inflammation responses, bioaccumulation). However, some toxic effects of carbon nanotubes are being related to the presence of metallic impurities remanescent from the synthesis process. Consequently, there is a need either develop impurity-free synthesis methods or improve the purification methods for CNTs before their contact with biological systems. Recent reports have shown the importance of using well characterized and purified samples of CNTs when evaluating toxic effects [77–80].

The hypothesis that CNTs could be biodegradable was firstly confirmed in a study that evaluated the interaction of SWCNTs (size from 200 to 900 nm measure through dynamic light scattering) with horseradish peroxidase (HRP), which induced a catalytic degradation of the nanotubes at a low hydrogen peroxide concentration. The study revealed the possibility of natural degradation of carbon nanotubes in the environment (Fig. 7.3) [81]. In this particular work, it was used SWCNTs produced through the electric arc discharge method and purified through oxidation by using H_2SO_4/H_2O_2 to remove metallic impurities from the catalyst. Oxidation also renders SWCNTs functional groups such as carboxylic acids. After an incubation period of 8 weeks, the average size of carbon nanotubes decreased to 231 nm and after 16 weeks of incubation no suspend particles was observed. Other techniques than dynamic light scattering (DLS) were used for supporting the results.

From this first report of CNTs biodegradation, other studies were performed in order to enlighten the phenomenon by using other techniques. It was reported that there are differences between the biodegradation of carboxylated (i.e. oxidized) and pristine SWCNTs when incubated with HRP. These results were compared with the chemical degradation of a model peroxidase (i.e. hemin and iron chloride). Authors reported that pristine SWCNTs did not have their structure altered by HRP whereas they showed that hemin and iron chloride can induce a significant degradation of SWCNTs [82]. Evidences also point out that when pristine SWCNTs are treated with HRP/H_2O_2 there is a heterolytic cleavage of hydrogen peroxide whereas with HRP nanotubes do not have any alteration of their chemical structure. Furthermore, the combination of hydrogen peroxide and iron chloride (i.e. Fenton catalyst) resulted in the cleavage of hydrogen peroxide that oxidized pristine SWCNTs. Explanation for the lack of oxidation in the system with pristine SWCNTs in contact with HRP is based on hypothesis that the ability of HRP-Compound I to degrade

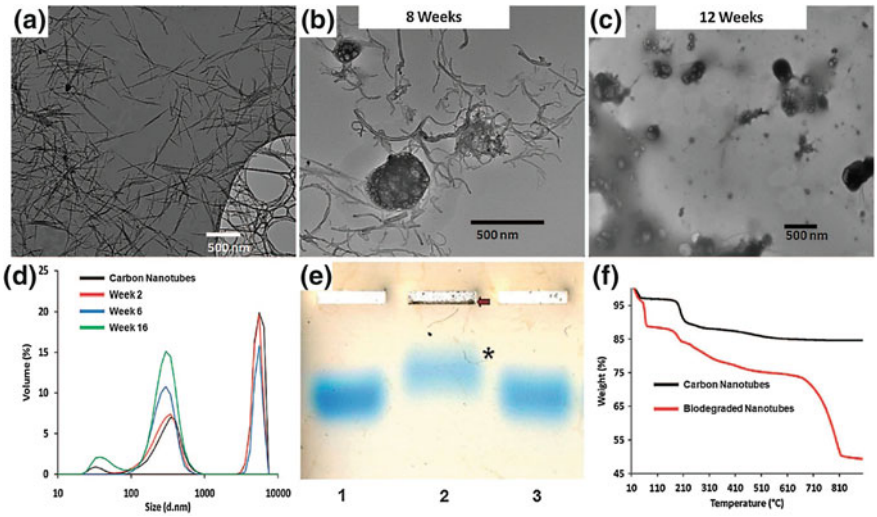

Fig. 7.3 Transmission electron microscopy (TEM) tracks the biodegradation of SWCNTs as a function of the time. (**a**–**c**) Length decrease is seen at week 8 with some globular material, while at week 12 mostly globular material is present. This change is attributed to the biodegradation of nanotubes. (**d**) Dynamic light scattering (DLS) shows size distribution in weeks. Larger material present in week 2 is not present in week 16, while additional smaller material is found to be present in week 16. (**e**) Agarose gel electrophoresis reveals that HRP/H_2O_2 functionalization results in the disappearance of nanotubes capable of binding albumin/bromophenol *blue* complex. (**f**) Thermogravimetric analysis (TGA) of carbon nanotubes prior to incubation (*black*) and after degradation (*red*). Reprinted with permission from [81]. Copyright 2008 American Chemical Society

SWCNTs depend on a ideal proximity between CNTs and the active heme site, which may not be achieved for this system (i.e. pristine SWCNTs and HRP) [82].

Another study compared the degradation capacity of different peroxidases (myeloperoxidase [MPO], HRP, hemoglobin, and lactoperoxidase [LPO]) in degrading oxidized SWCNTs (o-SWCNTs) purified by an oxidative treatment with H_2SO_4/H_2O_2. It was observed that the addition of the enzymes and H_2O_2 to the CNTs suspension leads to the inactivation of the enzyme active site due to the presence of H_2O_2, resulting in little degradation of o-SWCNT. The comparison of the ability of peroxidases to degrade o-SWCNTs indicated that MPO and LPO are more efficient, and this fact is possibly related to the ability of the former to generate hypochlorite species and the latter to produce hypobromite [83].

By mimicking the phagosome actions and inflammatory sites, the authors stepped towards a more realistic medium of biodegradation of CNTs. For this, o-SWCNTs were suspended and incubated with blood plasma at pH 5.8, containing high concentrations of the system MPO/H_2O_2 or hypochlorite. The efficient degradation of the nanotubes observed in the study is apparently related with the presence of hypochlorite species, which are possibly responsible for the oxidative degradation of carbon nanotubes in vivo [84].

In addition to the evaluations of biodegradation of SWCNTs, multi-walled carbon nanotubes (MWCNTs) were also considered, and this is relevant because most of the applications of carbon nanotubes that are in market are based on MWCNTs. The ability of HRP and hydrogen peroxide (H_2O_2) to chemically transform oxidized MWCNTs was also confirmed. Previously to these biological assays, pristine MWCNTs were sonicated in concentrated HNO_3 followed by a further sonication of these pre-treated MWCNTs (p-MWCNTs) in a mixture of H_2SO_4/HNO_3. The latter is responsible for providing carboxylated groups to the CNTs surface through an oxidation process (o-MWCNTs). Biodegradation of MWCNTs doped with nitrogen (n-MWCNTs, obtained through chemical vapor deposition [CVD]) was also tested [85]. Oxidized-MWCNTs obtained after 5 and 8 h of sonication with H_2SO_4/HNO_3 were partially degraded after 60 days of incubation with HRP/H_2O_2. These results were confirmed through a decrease in the light scattering and absorbance for both suspensions. For o-MWCNTs oxidized for 8 h, CNTs lengths were shortened from 1 μm to 100–400 nm. Pre-treated MWCNTs was found to be little transformed when compared with the oxidized nanotubes. The o-MWCNTs presented a faster CO_2 generation during the enzymatic process in comparison with pre-treated carbon nanotubes (p-MWCNTs). Analysis through transmission electron microscopy (TEM) showed that n-MWCNTs are complete degraded by the HRP/H_2O_2 system. This biodegradation was also confirmed through Raman spectroscopy, by following the evolution of D and G bands during enzymatic degradation. After the whole process, these bands are absent for n-MWCNTs. By considering the set of results, the authors suggested a layer-by-layer degradation mechanism, once nitrogen doping is responsible for increasing the density of defects in the graphitic structure [85]. The authors also support that the nitrogen doping provides binding sites for HRP enzymes during the oxidation process, which increase the degradation of n-MWCNTs.

More recently, it was published a report comparing the biodegradation effects in both SWCNTs and MWCNTs that were submitted to two different oxidative processes [86]. In that study, nanotubes with rather different morphologies were used: SWCNTs with lengths from 0.1 to 1 μm and diameter around 1 nm (sample 1); MWCNTs with lengths from 0.5 to 2 μm and diameters from 20 to 30 nm (sample 2); and MWCNTs with length around 1.5 μm and diameter about 9.5 nm (sample 3). After the oxidation treatment, samples 1, 2 and 3 exhibited 2.5, 1.7 and 1.9 carboxylic groups/mmol g^{-1}, respectively. In a first experiment, samples 1, 2 and 3 interacted with of an enzyme-free reaction medium, which is the phagolysosomal simulant fluid (PSF), and to the HRP/H_2O_2 system. SWCNTs underwent degradation by PSF after 30 days of incubation as well as by HRP/H_2O_2. The degradation capacity of latter mixture was already reported [87]. TEM analyses showed for samples 2 and 3 the existence of morphological and structural changes of MWCNTs mainly when they were incubated with the HRP system. Authors suggested that carbon nanotubes degradation mechanism differs from SWCNTs to MWCNTs. Multi-walled carbon nanotubes appears to be extensively shortened rather than unzipped, which occurs mainly for SWCNTs. There were evidences that MWCNTs are also peeled off, resulting in pieces and debris fragments [52, 86].

Summarizing, there are clear evidences that both SWCNTs and MWCNTs can be degraded in the presence redox-enzymes, in particular the system consisting of HRP/H_2O_2. Other peroxidases such as myelo- and lacto- as well as hemin system can also degrade CNTs. Furthermore, Fenton catalyst (i.e. $FeCl_3$+H_2O_2) and some simulated biological fluids such as phagolysosomal simulant fluid were also capable to degrade CNTs in certain specific conditions. By considering that CNTs can be currently though as a large class of carbon nanomaterials that can be produced and processed through several methods, these biodegradation results are very important in the sense that they can drive the scientific community and industry towards the generation of CNTs with specific combinations of morphology, size, chemical structural and surface characteristics, in order to achieve bio-friendly products.

7.4.2 Cytotoxicity of Carbon Nanotubes

Digestion of SWCNTs by primary monocytes, macrophages, microglia and dendritic cells can be initiated through the functionalization of CNTs with phosphatidylserine coatings, which appears to offer an initial signal for the digestion. These results were observed for SWNCTs produced through a high pressure CO disproportionation process, which employs CO in a continuous-flow gas phase as the carbon feedstock and iron as the catalyst precursor. Previously to the in vitro assays, purification of the products was further carried out through an acid treatment to remove metal residues, thus resulting in CNTs with sizes varying from 130 to 180 nm and a final concentration of metallic residues of 0.23 %-weight. A similar degradation was observed in vivo by the action of alveolar macrophages. These strategies to improve target and uptake of CNTs by phagocytes are extremely beneficial and are considered as the initial step to enhance biodegradation of CNTs in different contexts of applications [88]. For this purpose, the authors must focus on the interaction of CNTs with lysosome, which has been considered the intracellular site for degradation of these nanomaterials [89].

Another important approach considered the possibility of peroxidase intermediates inside human cells or fluids to be related to the degradation of SWCNTs [87]. It was observed that reactive radicals from hypochlorous acid and from the human neutrophil enzyme myeloperoxidase are able to catalyze the biodegradation of SWCNTs in vitro. The degradation was observed mainly for neutrophils whereas macrophages induced it to a lesser extent. It was also observed a non-toxic behavior of biodegraded SWCNTs, which correlates the degree of degradation to the degree of the associated inflammatory response in a possible organism exposed to CNTs. In future studies, it is necessary to correlate the SWCNTs characteristics (i.e. length, diameter, purity, degree of functionalization) to these biodegradation process observed by neutrophils.

SWCNTs (70–90 % of purity) bio-persistence was evaluated in vitro by simulating for 90 days the phagolysosomal medium, which contains oxidants including hydrogen peroxide, superoxide anion and hydroxyl radical, species responsible by

the microbial digestion. Firstly, SWCNTs were carboxylated and shortened through oxidation with a mixture of concentrated sulfuric and nitric acids (H_2SO_4/HNO_3). It was observed that carboxylic functions and defects on the surface of SWCNTs increase the CNTs biodegradation. In addition, unfunctionalized CNTs and CNTs modified through other processes of surface functionalization such as ozonolysis, annealing (at 1000 °C) and aryl-sulfonation did not undergo morphological variations during the period of incubation (90 days) in phagolysosomal conditions [90].

Biodegradation of double-walled CNTs (DWCNTs) was evaluated for nanotubes with purity higher than 60 %, length from 1 to 5 μm, synthesized by the combustion chemical vapor deposition method (CCVD) by using iron as the catalyst. DWCNTs were also treated in concentrated nitric acid for purification and further oxidized in a mixture of nitric and sulfuric acids [91]. By following the evolution of radial breathing modes (RBM) of DWCNTs through Raman spectroscopy in single cells, it was evidenced the intracellular modifications of the nanotubes. Biodegradation led to the formation of defects firstly on the outer surface of the nanotubes after their internalization in human prostate adenocarcinoma (PC-3) and human cervical carcinomas (HeLa) cells. The chemical structure of DWCNTs inner wall was preserved up to 12 h of incubation, when the RBM signal starts to decrease.

The possible fate of CNTs after their administration was also studied by using amino-functionalized MWCNTs (MWCNTs-NH_3^+). The study used MWCNTs with 50 nm of diameter and 200 nm of length, which were incubated with phagocytic macrophages of RAW 264.7 murine and non-phagocytic A459 human lung carcinoma cells in the presence of different well-known active uptake inhibitors. The hypothesis was that after cellular internalization, CNTs fate could be influenced by their ability to cross cellular membranes. In fact, in a short time free carbon nanotubes were observed in the cytoplasm after their cell incubation, indicating that MWCNTs-NH_3^+ were able to escape from phagosomes, which prevented their elimination by endo-lysosomal or/and phago-lysosomal vesicles. Furthermore, it was evidenced the formation of wrapped CNTs into endosome-like structures after 12 h of incubation, possibly due to the formation of endo-lysosomal vesicles which contains enzymes, thus leading to an enzymatic degradation [92].

Toxicity of SWCNTs on the immune system, specifically on primarily cultured murine peritoneal macrophages and purified splenic T cells was also studied. SWCNTs used had purity >90 %-wt and ash residues <1.5 %-wt; were synthesized by the chemical vapor deposition (CVD) method; and were oxidized by a mixture of H_2SO_4/HNO_3 for 24 h at 40–50 °C [93]. Both as-synthesized and acid-functionalized SWCNTs (AF-SWCNTs) had diameters from 1 to 2 nm and an average length of 150 nm. Exposure of macrophages to 0–50 μg mL^{-1} of both SWCNTs induced no significant cytotoxicity. However, analyses of the mitochondrial membrane potential and proteasome subunit gene expression showed that AF-SWCNTs (incubated with concentrations from 10 to 50 μg mL^{-1}) induced damages to mitochondrial function and proteasome formation, as a function of the concentration used. Phagocytic cells were affected at a level of 20 μg mL^{-1} for both as-synthesized and acid-functionalized SWCNTs. Nevertheless, AF-SWCNTs

(5 μg mL^{-1}) acted towards the inhibition of the phagocytic efficiency of latex beads in macrophages and also influence on the differentiation of naive T-cell to Th1 types (IFN-γ and TNF induction), implying risk of diseases related to Th1. Authors also evidenced that the lengths of AF-SWCNTs found in lysosomes/phagosomes were shortened by a 6-fold ratio. This result is in agreement with other studies that studied the degradation of carbon nanotubes by myeloperoxidase (MPO) in macrophages and neutrophils [87]. Consequently, considering results obtained, authors suggested that the CNTs shortening in macrophages may be a result of a selective uptake of AF-SWCNTs, once size-dependent cellular uptake of SWCNTs occurred, as previously demonstrated in NIH-3T3 cells [94]. Furthermore, the biodegradation of CNTs by macrophages under these conditions is, in part, a defense mechanism and it could decrease potential toxic effects of these nanomaterials [93].

In vitro studies also showed evidences that carbon nanotubes can be biologically degraded. These studies on the interaction of CNTs with immune cells that focused on cellular recognition of nanotubes and their enzymatic degradation are essential to establish the safe applications of these nanomaterials. Another important aspect that is related to the extent of the carbon nanotube biodegradation is the dependence on their surface microchemical environment, which can be largely modified by functionalization processes. In this context, the functionalization of carbon nanotubes with oxygenated groups and the introduction of defects on the material surface appear to facilitate enzyme biodegradation. In fact, defects on the carbon nanotube surface also increase its accumulation in human cells, increasing their internalization. Furthermore, some enzymatic systems such as lysosomal enzymes can act as oxidants of carbon nanotubes in cells.

7.4.3 Graphene, Enzymes, Microorganisms and Cells

Graphene has been rapidly introduced to the context of nanobiotechnology due to its potential in several fields of application such as a biosensing, for the detection of biological molecules [95]. However, as graphene has been produced more recently in comparison to carbon nanotubes [3], nanotoxicological research on this carbon nanomaterial has been less exploited. Regarding the toxicological aspects of graphene (e.g. biodegradability, biopersistence, cytotoxicity, long-term biological fate), experimental protocols which have been used so far to evaluate carbon nanotubes bio-effects are now being applied to this other nanocarbon material.

By using the same approach applied so far for CNTs, it was reported the evaluation of the capacity of HRP to degrade graphene-based nanomaterials. Graphene oxide (GO) which was obtained from graphite oxide was used for the study. Graphite oxide is diluted (1:4) with distilled water and exfoliated for 30 min through sonication, followed by 30 min of centrifugation to remove unexfoliated sheets, leading to GO sheets of about 0.61 nm high. It was observed that at low hydrogen peroxide concentrations, HRP is able to catalyze the oxidation of

graphene oxide (∼0.125 %-wt concentration); and this degradation leads to the formation of holes in its basal plane. Furthermore, this enzymatic process did not affect the reduced form of graphene (rGO) in the extent of its oxidized form (GO). Computational modeling showed that HRP preferentially bonds to the basal plane rather than the edge of both graphene oxide and reduced graphene. In this way, GO is more susceptible to carbon-carbon bond cleavage, thus facilitating the degradation at its basal planes [96].

In the particular case of graphene, not only bio-oxidation mechanisms were studied, but also bio-reduction. This aspect is justified by the possible application in electronic devices of graphene rather than GO. Consequently, electronic industry will demand a large-scale production of graphene and there is a huge interest in the reduction of graphene oxide obtained from the exfoliation of graphite, being the latter a largely available raw-material. In this way, it was observed that graphene oxide, produced from graphite powder using the Hummers method can act as a terminal electron acceptor for metal-reducing and heterotrophic bacteria present in the environment [97]. Electron transfer mediation to graphene oxide was performed by cytochromes MtrA, MtrB, and MtrC/OmcA, while mutants lacking CymA, another cytochrome associated with the extracellular electron transfer still retain the ability to reduce graphene oxide. These findings confirmed that graphene oxide can be biotransformed under environmental conditions.

Another green approach for the reduction of graphene oxide was recently reported. Bi-layered GO with a thickness around 1.7 nm was produced by a modified Hummers method [98], which used natural graphite as the raw material. A procedure which makes use of wild carrot root with endophytic microorganisms was able to reduce exfoliated graphene oxide to graphene (rGO) in aqueous medium at room temperature. Characterizations by different techniques demonstrated the removal of oxygen-containing functional groups from the surface of graphene oxide, thus resulting in the formation of graphene single layers with surface defects [99].

Toxicological aspects associated to the apoptosis and cytotoxicity induced by graphene in normal human lung cells (BEAS-2B) indicated a concentration- and time-dependent decrease of the cell viability (concentrations from 10 to 100 μg/ml) after 24 and 48 h of exposure. It was also observed an increase in early and late apoptosis of cells compared with controls [100]. In addition, graphene induced cytotoxicity and mitochondrial injury in human neuronal cells (PC12 cells) in a shape- and dose-dependent manner [101]. However, the reduced form of graphene oxide (rGO) interacting with human hepatoma cells (HepG2 cells) exerted only a moderate effect on protein levels [102]. In a useful comparison, the cytotoxicity level of graphene and carbon nanotubes was evaluated on neuronal PC12 cells [103]. In this particular case, it was found that toxicity was composition- and shape-dependent, with graphene exhibiting an overall lower toxicity than CNTs. Oppositely, in other study the toxic-effect of graphene was found to be inversely proportional to the concentration [104], being more toxic than CNTs at low concentrations.

Along with morphological variations, functionalization of graphene can strongly alter its cellular toxicity. Interaction of a human monocytic leukemic cell line (THP-1) with the reduced form of graphene oxide (rGO) and bovine serum albumin (BSA) led to the production and release of inflammatory cytokine (IL1B) [105]. There was a significant decrease in rGO cytotoxicity by the presence of functionalizing molecules on its surface. Recent results also indicate that the cytotoxicity of GO capped with polyethylene glycol (PEG) is significant reduced by the presence of this functionalizing agent. In this context, it was showed that incubation of PEGylated GO with colon cancer cell lines (HCT-116), human ovarian carcinoma cell line (OVCAR-3), lymphoblastoid cells (RAJI), glioblastoma cell line (U87MG), and breast cancer cells (MCF-7) did not lead to cytotoxic effects in GO concentrations of up to 100 μg mL^{-1} [80]. PEGylation is the most used method to improve the solubility and biocompatibility of nanomaterials for applications in biomedicine. Nanographene oxide functionalized with polyethylene glycol (NGO-PEG), produced through a modified Hummer's methods (size ~ 5 to 50 nm) was successfully used for the delivery of the hydrophobic (water insoluble) cancer drug SN38, thus leading to a soluble NGO-PEG-SN38 complex [106].

Regarding the capacity of macrophages to internalize and remove graphene-based materials, it was observed that phagocytic cell lines were able to internalize nano- and micronized GO with different lateral sizes, thus showing a selective internalization [107]. GO had no toxic effect to these cells in an uptake of up to 20 μg/mL. The study revealed that the presence of manganese impurities on GO increases the cell toxicity, indicating the importance of purification of the material. Furthermore, considering the uptake of the phagocytic cell lines, two different sizes of GO were internalized via different initial cell interactions [107]. This toxic effect associated with the manganese impurities is an important aspect to be considered for all nanostructures, once these impurities could cause inconsistent conclusions from the nanotoxicity studies.

7.4.4 Biodegradation and Cytotoxicity of Fullerenes

Fullerenes present a great potential for biomedical applications, in particular as anticancer nanomaterials, mainly due to their capacity to generate singlet oxygen species that act as radical scavengers, thus resulting in an efficient antioxidant agent [23]. Along with the development of fullerene biomedical potentialities, there were several concerns regarding the toxicity of this nanostructure once it was reported that pristine C_{60} could have a very long biological half-life [24]. In this way, biotransformation of fullerene and its derivatives was largely studied. In this context, laccase was successfully used in the biotransformation of fullerene (C_{60}, 99.9 %, diameter around 1 nm). Laccase complex systems (dendritic copolymers/laccase/mediator N-hydroxy-5-norbornene-2,3-dicarboxylic acid imide/1-hydroxybenzotriazole or 2,20-azinobis (3-ethylbenzothiazoline-6-sulfonic acid)/1-hydroxybenzotriazole)

were the responsible for the biotransformation of fullerene into epoxide- and hydroxyl-derivatives, under mild and environmental friendly reaction conditions.

Biotransformation of fullerene nanowhiskers (C_{60} NWs) was also evaluated. They were synthesized by a liquid-liquid interfacial precipitation method using a C_{60}-saturated solution in toluene and isopropyl alcohol. The method was able to produce nanowhiskers with an average length around 6.0 μm and average diameter of 660 nm. TIP-1 macrophage-like cells treated with phorbol 12-myristate 13-acetate were exposed to 10 μg mL^{-1} of fullerene nanowhiskers (NWs). Results showed internalization larger than 70 % for C_{60} NWs after 48 h of exposure. After incubation, degraded NWs were found in cells and the number of NWs shorter than 3.0 μm (length) increased after the exposure. It was suggested by the authors that macrophages could be able to decompose C_{60} NWs into molecules of C_{60}. In addition, the presence of granular particles was observed after the interaction with fullerene whiskers. Possibly this is a result of the re-crystallization of C_{60} molecules via processes of dissolution and recrystallization, during the long-term co-culture or upon the enzymatic treatment [108]. Later, these same authors observed very small effects caused by C_{60} NWs on the cytotoxic activity and induction of gene expression on the human acute monocytic leukemia cell line THP-1, as compared to MWCNTs. It was suggested that THP1-treated cells were able to disaggregate C_{60} NWs into fullerene molecules, which result in small effects on the cytotoxic activity due to a smaller capacity to induce cellular gene expression, which can be associated to the biodegradable property of C_{60} NWs in cells [109].

Complementarily, a cytotoxicity study of fullerenes on human derived macrophages (monocytes) indicated no toxic effects in in vitro experiments. C_{60} dispersions (99.9 %) were prepared using THF and mixed to a serum-free cell culture medium. The C_{60} dispersion in THF showed the presence of single and poly-crystals with varying shapes and sizes (particles diameters from 60 to 270 nm and clusters diameters from 420 to 1300 nm). It was observed that C_{60} crystals aggregate within lysosomes (possibly due to their internalization by phagocytosis or endocytosis) in which C_{60} was degraded into smaller structures [110]. Possibly, this is an indication that aggregation or disaggregation phenomena that occur within the cells may be ruled by some enzymatic processes.

C_{60}-fullerol biodegradation was also evaluated. The compound was synthesized from C_{60} (99 %) and KO_2 in CH_2Cl_2 in the presence of 18-crown-6, forming a brown precipitate that was further purified by washing the products with CH_2Cl_2, and finally dried. It was evidenced by the authors that two white rot basidiomycete fungi (*Phlebia tremellosa* and *Trametes versicolor*) metabolized and degraded C_{60}-fullerol. Both fungi produce oxidative enzymes such as lignin peroxidase, manganese peroxidase, and laccases, which oxidize complex molecules such as lignin as well as polycyclic aromatic compounds [111, 112]. After 32 weeks, both fungi species could degrade fullerol as well as oxidize a small portion of it to CO_2, and incorporate it into fungal biomass [113].

Results presented so far by the scientific community on the interactions between carbon nanomaterials and cells reveals the necessity of a better understanding of the

cell uptake and trafficking which are directly related to the biotransformation of these nanomaterials. Regarding the processes of cell internalization, it is currently known that endocytosis is the most common way of incorporating nano-objects into cells by enclosing them in vesicles formed by the cytoplasmic membrane. Endocytosis comprises three mechanisms: pinocytosis, phagocytosis, and caveolae-dependent or clathrin-mediated endocytosis [62]. Although there were studies which tried to correlate morphological and chemical characteristics of carbon nanomaterials (e.g. surface functionalization, length, diameter) to the occurrence of specific mechanisms of endocytosis, until now, much data reported from different groups are sometimes inconsistent [114]. Furthermore, by knowing that the cellular localization of carbon nanomaterials also depends on the internalization pathway, morphology and physicochemical characteristics [115], the trafficking of these nanomaterials is so far an object of intense debate, and a lot of work has to be done to elucidate this issue.

7.5 In Vivo Toxicity of Carbon Nanomaterials

7.5.1 Carbon Nanotubes

In vivo toxicity of carbon nanotubes has also been studied along the past years. In one of these studies, SWCNTs (diameters from 1 to 4 nm; produced by the high pressure CO disproportionation process [HiPco]) were administered into myeloperoxidase knockout B6.129X1-MPO (MPO k/o) and wild-type C57Bl/6 (w/t) mice [88, 116]. After 28 days of exposure, it was observed large differences between the Raman D-band/G-band ratios of SWCNTs, which indicated a higher SWCNTs degradation in w/t mice compared to MPO k/o animals [116]. In addition, it was evidenced that the oxidation and elimination of SWCNTs from the lungs of these animals (MPO k/o) after pharyngeal aspiration occurred in a lesser extent compared to wild-type C57Bl/6 mice, whereas the inflammatory response was higher. These results provided direct evidence of the participation of MPO, a key enzyme in the inflammatory response, in the in vivo pulmonary oxidative degradation of SWCNTs.

Another study compared differences in toxicity between SWCNTs and MWCNTs, synthesized by using arc-discharge evaporation of graphite rods. SWCNTs had 2–3 nm of diameter and 30–50 nm of length, and the MWCNTs had 5–20 nm of diameter and were 300–2000 nm long. The chemical structure of these nanotubes ($\sim$99.5 %) was studied by Raman and FTIR spectroscopies, thermogravimetric analysis, and electron microscopy before their introduction into living systems. MWCNTs aggregated within living tissues whereas SWCNTs were easily phagocytized by macrophages and transported to local lymph nodes. Metabolic processes were estimated in the regenerating fibers by histoenzymatic staining, and the detection of oxidative enzymes confirmed the authors' histological observations [117]. Conclusions were taken towards the existence of an oxidative degradation of single-walled carbon nanotubes with very little aggregation.

Considering the promising perspectives of CNTs in neurological applications such as implants or drug delivery carriers, an investigation of the distribution and fate of CNTs within the neuronal tissue was performed. CNTs functionalized with amino groups (NH_3^+) were introduced into the mouse brain cortex and monitored by analyzing through transmission electron microscopy and Raman spectroscopy the brain tissue sections for a period from 2 to 14 days after the administration. It was used MWCNTs with 94 % of purity and with outer diameters from 20 to 30 nm, and lengths from 0.5 to 2 mm. At initial periods after application it observed the occurrence of a large structural deformation of CNTs, leading to a partial degradation of MWCNTs-NH_3^+ in vivo, following by the internalization within microglia. With the results, authors called the attention for the necessity of more studies evaluating the effects of CNTs on brain tissue [118]. They also suggested the use of high-resolution equipments towards the understanding on the CNTs biodegradation in the brain.

7.5.2 Fullerenes

In vivo toxicity of fullerenes (sand grain-like particles with sizes of up to 50 micrometers) was evaluated on p53-heterozygos (p53(±)) mice administrated through intraperitoneal applications. It was observed only small brownish black plaques on the serosal surface. Histological analyses also indicated the presence of plaques containing polygonal clefts and lacunae surrounded by a thin layer of foamy cells and separated by thin fibrous septa. Clefts/lacunae sizes and shapes are well correlated to the morphology of injected fullerene aggregates. Furthermore, it was observed that the edge of the clefts was brown pigmented, indicating a possible biodegradation of fullerene by phagocytic cells, proteins and/or other organic components [119].

Analogously, another work employed fullerene of 99.98 % of purity (containing 7 % of particles with diameter between 1000 and 1650 nm; 43 % between 500 and 1000 nm; 28 % between 250 and 450 nm and 22 % below 200 nm). Intraperitoneal administration of fullerenes in mice presented a maximum accumulation of fullerenes, reaching about 24 % of the injected amounts, during the first week after the injection. At day 14 and day 21, the medians of the concentrations decreased to 5 and 1 % of the values measured at day 7, respectively. The results indicate that C_{60} could be eliminated and/or transformed by the rat livers [120].

7.5.3 Graphene

In vivo studies based on tissue distribution and elimination of graphene are still very limited compared to CNTs and fullerenes. However, it is already known that administration of GO in mice can induce chronic toxicity and lung granulomas

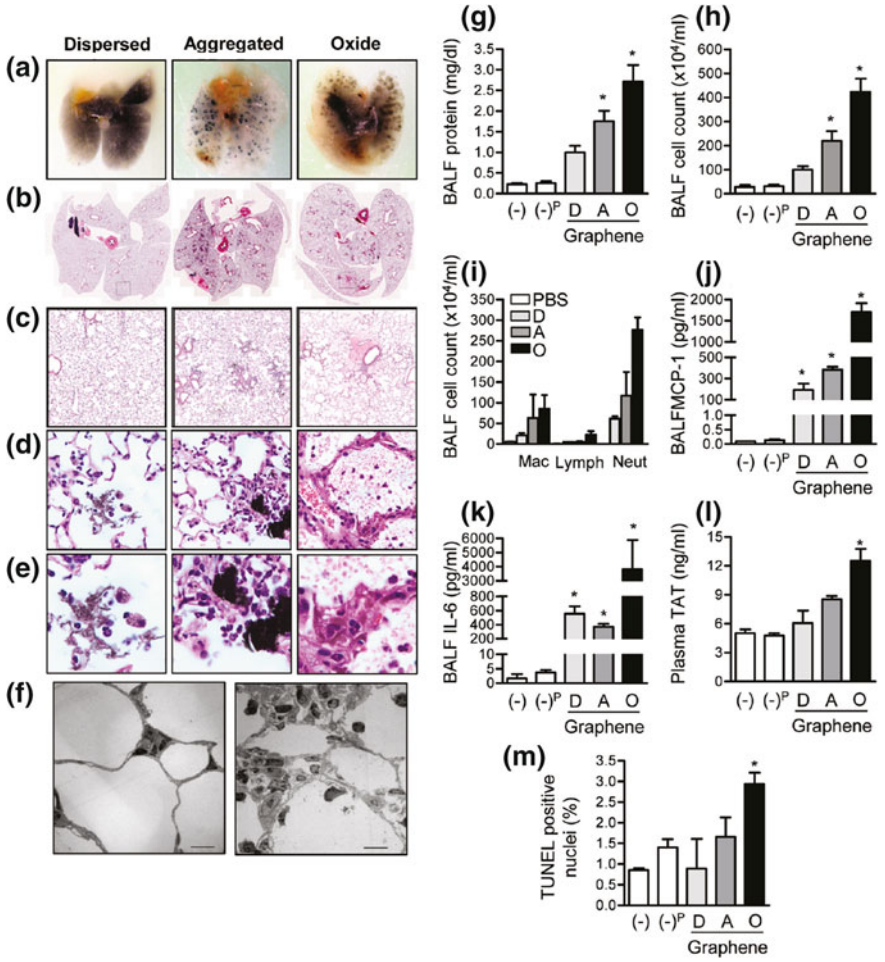

Fig. 7.4 Graphene oxide induces acute lung injury in mice. Mice were treated intratracheally with three preparations of graphene: highly dispersed and purified in 2 % Pluronic (Dispersed, D), aggregates suspended in water (Aggregated, A) or GO (Oxide, O) and appropriate controls, water (-) or 2 % Pluronic $(-)^P$. Twenty-four hours later the mice were sacrificed for assessment of lung injury. **a** Photomicrograph of paraffin blocks of the lung after sectioning at approximately the same level demonstrating the distribution of particles. **b–e** Photomicrographs of lung sections at <1× (**b**), 50× (**c**) and 200× (**d**). **f** Representative electron micrographs from lung sections of mice treated 24 h earlier with Pluronic-dispersed graphene (*left*) or GO (*right*), bar indicates 10 μm. Bronchoalveolar lavage fluid (BALF) levels of (**g**) protein, **h** total cell count, **i** differential cell count Macrophages (Mac), Lymphocytes (Lymph), Neutrophils (Neut) and levels of the pro-inflammatory cytokines (j) MCP-1 and (k) IL-6. In the same animals, (l) plasma levels of thrombin-antithrombin (TAT) levels and (m) the percentage of TUNEL positive nuclei in paraffin embedded lung sections. Reprinted with permission from [122]. Copyright 2011 American Chemical Society

[121]. Furthermore, a dose-dependent pulmonary toxicity, granulomatous lesions, pulmonary edema fibrosis and inflammatory cell infiltration were also observed for this nanomaterial (Fig. 7.4) [122, 123]. Pulmonary inflammatory responses were also observed for graphene coated with bovine serum albumin (BSA) administrated in rats [105]. Oppositely, toxicological studies which focused on the uptake of PEG-coated graphene nanosheets in mice and subsequent photothermal treatment of cancerous tumors did not indicate any adverse toxic effect [104, 124]. PEGylation of GO apparently reduces the toxic effects of graphene oxide on mice once no severe toxicity was measured in vivo upon administration of GO as a component in injectable hydrogels for tissue engineering [125]. Regarding the fate of GO and PEGylated GO after oral feeding and intraperitoneal injection into healthy mice [126], it was observed that PEGylated GO present no uptake via oral administration, indicating limited intestinal absorption, with almost complete elimination. In contrast, upon intraperitoneal injection in mice, PEGylated GO was found to accumulate in liver and spleen [126].

7.6 Influence of the Chemical Structure and Surface Microchemistry on Nanotoxicology

Carbon nanomaterials, more specifically CNTs, fullerenes and graphene based-materials currently impose nanotoxicology great challenges mainly in the sense of standardizing models and protocols for toxicity assays, once little alterations in their chemical structure or surface can strongly influence on biological interactions. This is one of the main causes of the lack of efficient models for elucidation of interaction and biodegradation of carbon nanomaterials in biological systems. So far it was observed that surface charge interferes in the defense response of human organism once macrophages can absorb more easily negatively charged SWCNTs compared to non-functionalized SWCNTs; the latter do not induce the production of nitric oxide [127, 128].

In addition, surface treatments or surface functionalizations with oxygenated groups replacing sp^2 carbons in the graphitic network of NTCs (e.g. through oxidation or doping) may lead to changes or defects on the surface of these nanostructures that appear to interfere directly in the process of their biodegradation [81, 82, 83, 84, 85, 86, 87, 91, 93, 116, 129]. However, the mechanism by which oxygen groups such as alcohols, ketones, ethers, esters, carboxylic acids and anhydrides, and other oxidized shorter graphitic-based fragments affect the degradation is not yet elucidated. There are evidences that possibly the breaking of the polyaromatic structure of the graphite sheets is directly related to the degradation of these carbon materials. CNTs, fullerenes and graphene-based materials when in pure form and without any surface treatment present a high hydrophobicity, which often restricts their individualization and dispersion in complex biological organism such as cells, cell organelles as well as their ability to interact with redox-enzymes

that could perform degradation. Therefore, it is of fundamental importance that the toxicology assays consider precisely their solubility, morphology, particle size and also their ability to form agglomerates or clusters [99, 109, 110, 117].

For CNTs, graphene and fullerene, assuming that all of them can somehow be individualized dispersed in the biological media (e.g. by chemical functionalizations), graphene and carbon nanotubes exhibit a very distinct behavior from that of fullerene, which has a typical molecular characteristic, with well defined chemical structure and morphology. Nanotoxicology thus must take into account in its protocols and models that CNTs and graphene have significant size and morphological variations among the different samples used around the globe. Furthermore, surface microchemical environment of these carbon nanomaterials (CNTs and graphene-based) can also possess very different characteristics depending not only on the synthetic methods used for their production, but also on chemical reactions performed in the functionalization processes. In this way, it is imperative to define the morphology (e.g. length, thickness, diameter, and aspect ratio) and the chemical characteristics of the surface previously to be submitted to toxicological assays, once small changes in these parameters may lead to a significantly different biological effect.

The morphological influence on the bio-nano interactions of carbon-based nanomaterials, largely evaluated mainly for carbon nanotubes [86], evidences indicated that the surface chemistry of these materials is playing a key role over the bio-nano interaction. By considering the surface charge, it was observed that negatively-charged functionalized SWCNTs (i.e. oxidized SWCNTs) are more readily uptaked by macrophages [127] in comparison to non-functionalized SWCNTs, being the latter poorly recognized, and they do not induce common activation responses (e.g. production of superoxide and nitric oxide (NO)) [128]. Surface functionalization with oxygenated groups in the sp^2 graphitic structure had showed to influence substantially on the biodegradation of these materials by enzymes, cell or microorganisms [81, 82, 83, 85, 86, 87, 90]. These oxidation processes are the main functionalizations performed in CNTs and graphene when biological applications are aimed. From a detailed observation, the surface microchemical environment of oxidized carbon nanotubes and graphene contain a variety of oxygenated groups such as alcohols, ketones, ethers, esters, carboxylic acids and anhydrides randomly located over the surface [50, 130]; and other shorter oxidized fragments which can be stabilized through strong adsorption on preserved graphitic sheet regions [50, 78]. Although a detailed analysis on how each oxygenated group is influencing the bio-nano interactions of these nanomaterials is difficult to be performed, their presence must be considered and oversimplifications must be avoided towards a better comprehension of their roles. Other processes able to induce surface defects besides oxidation (e.g. nitrogen doping) were seen to be efficient to provide biodegradability features for CNTs [85], thus supporting the idea that the degradation is directly related to the breaking of the polyaromatic structure of the graphitic sheet.

When nanotoxicology is dealing with pristine carbon nanotubes, graphenes and fullerenes in their pure form, with preserved polyaromatic chemical structures, their low solubility in aqueous biological media impose the use of other parameters such

as powder granulometry and agglomeration state, in order to understand the bio-nano interactions manifested. It has been reported that the agglomeration state of pure carbon nanotubes [117], graphene [99] and fullerenes [109] has a direct impact in the way enzymes, cells and cellular organelles interact with them.

Along with the morphological, chemical and surface microchemical variations from synthesis, processing and functionalization methods used for the carbon-based nanostructures, there are also variations to be considered in results obtained from different characterization protocols employed around the globe, fact which has been already revealed by an interlaboratorial studies [131]. Therefore, nanotoxicology has the imperative task of standardizing the samples and the protocols for experimental analyses, once interlaboratorial studies and comparisons indicated a significant difference of characterization results (i.e. size of nanoparticles and zeta potential) for the same sample analyzed in different laboratories around the globe. Thus, an optimization of both synthesis and characterization methods used for carbon-based nanomaterials is important for advancing in the understanding of their toxicity as well as for standardizing the protocols [132,133].

7.7 Conclusions and Perspectives

The influence of nanotechnology in biology and in medicine, in particular, has been different from all other areas of knowledge because of a main reason: there are complex interactions that occur in a particular biological process, in which, undoubtedly, nanomaterials should interact and maintain the integrity of the whole process and should not damage any other parallel process. To ensure this, extensive studies are needed to confirm the effectiveness and non-toxicity of the materials used.

Besides the fact that nanotechnology is becoming a mature field with great advances made so far, there is still a plenty of room for the nanotoxicology to develop and provide the scientific basis for the safe use of nanomaterials. More than this, nanotoxicology is crucial for the sustainable development of nanotechnology in a broad sense. Inexorably, processes of creation of nanomaterials and the study of their biological application require broad approaches on the construction of matter, not restricted to the concepts from basic theories of chemical bonding established throughout the twentieth century. Considering the very nature and peculiarities of nanomaterials which were described here, and also the dynamics related to the biological mechanisms occurring in several degrees of complexity, the correlation between the supramolecular interactions occurring at the bio-nano interface and the consequent bio-effects manifested is currently the biggest challenges toxicology. However, all these difficulties are not preventing researchers to achieve important advances.

Since carbon nanostructures are front runners in nanotechnology due to their richness of new phenomena as well as their promising proprieties which could be used in real world applications, they should be also analyzed in terms of safety for both humans and environment. However, the development of a standard metrology for

these materials is one of the first barriers to be overcome, previously to the assessment of toxicological effects. Furthermore, by considering the overall functional cycle of a particular biological process, its interaction with carbon nanomaterials is very complex and the elucidation of this interaction involves a grand challenge in the field. Currently, the studies of this interaction requires the development of new methods and techniques for monitoring nanoparticles in all steps of interaction, involving internalization mechanisms, migration within the cell and interaction with different organelles, penetration in the nucleus, translocation among tissues and organs, and the elimination of the nanoparticle. In particular, in the case of carbon nanotubes and graphene, which have very strong optical properties, these properties are very sensitive when the nanostructures interact with environment. In this way, the use of optical techniques such as resonance Raman spectroscopy and photoluminescence combined with near field optical techniques can lead to new tools and methodology for analyzing these carbon nanostructures in the context of nanotoxicology. These techniques can provide a very detailed characterization of the nanotubes (diameter, chirality, edge structure, defects, charge, etc.) and could probe them individually (just one nanotube or a piece of graphene). By combining them with standard biomarkers it is possible to correlate the physico-chemical properties of carbon nanostructures and the bio-effects manifested in biological system with several degrees of complexity.

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Chapter 8
Synthesis, Purification and Functionalization of Carbon Nanotubes for Biotechnological Applications

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Abstract This chapter addresses the initial steps one has to give to explore the unique properties of carbon nanotubes in biomedical applications, which are their synthesis, purification and functionalization. We start with a brief description of the structure and properties, and we summarize the synthesis methods. The major issues are then addressed, which are purification and functionalization for bioapplications. We review the different methods, highlighting their advantages and disadvantages.

8.1 Introduction

The lack of solubility in water and physiological media, and the tendency to form tangled aggregates are the initial obstacles to be overcome in order to explore the unique properties of carbon nanotubes (CNTs) in bioapplications. This requires the

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use of chemical synthetic methods in order to make them soluble and to impart them with tuned surface functionalities. CNTs are, however, a complex class of nanomaterials. Characteristics such as the number of walls, length, inner and outer diameters, degree of graphitization, types and concentration of defects are directly dependent on the growth method and subsequent purification procedures. The purification is essential to eliminate catalytic and carbonaceous impurities and to reveal the actual nanotube surface. The choice of chemical routes for further modifications has to be made from a detailed knowledge of the starting material. After more than two decades of study, it is now well defined that the interactions between CNTs and biological systems will be dictated by the structural characteristics, the surface composition of the CNTs and by the nature and content of impurities. The biological interactions of CNTs may be either beneficial or harmful, and the biological response may be influenced by the biologically effective dose and the physicochemical properties and purity of the nanomaterials. The control of the latter is sought mainly through their synthesis, purification and functionalization, which are the focus of this chapter.

8.2 Carbon Nanotubes: Structure, Properties and Synthesis

8.2.1 Carbon Nanotube Structures and Properties

Discovered in 1991 [1], CNTs are considered one of the most remarkable examples of nanomaterials. They are an important object in the study of one-dimensional systems in basic and interdisciplinary science, and have important applications, especially in the areas of molecular electronics, composites, energy generation and storage, environmental remediation and nanomedicine.

CNTs are cylindrical structures formed by carbon atoms arranged in a hexagonal lattice with a high aspect ratio and a diameter in the nanoscale range [1]. They can be classified as to their structure into two main types: single-walled carbon nanotubes (SWCNTs) and multi-walled carbon nanotubes (MWCNTs). SWCNTs may be thought of as being formed by a rolled graphene sheet, with each end closed by half of a fullerene molecule. There are several ways to roll a graphene sheet in a way that two crystallographically equivalent sites of its hexagonal lattice coincide to form nanotubes with defined geometry. Thus, CNTs can be formed in three different geometries: zigzag, armchair or chiral. MWCNTs comprise multiple layers of concentric tubes with a space of about 0.34 nm between adjacent layers [2].

The combination of the parameters chiral angle (θ) and diameter (d_t) defines the electronic character of each CNT. All armchair tubes ($\theta = 30^o$) are metallic, while zigzag ($\theta = 0$) and chiral ($0 < \theta < 30^\circ$) tubes can be either conductors or semiconductors, with gaps ranging between 1.8 and 0.5 eV [2]. Due to their high aspect ratio, the electronic transport in metallic nanotubes is ballistic, i.e. it occurs without scattering, which enables current conduction through large areas of an unheated tube.

CNTs have low capacitance, strong optical response and are good photon emitters (CNT semiconductor), as they have a direct bandgap [3]. CNTs are also great thermal and electrical conductors. Their current density is 1000 times greater than that of metals like silver and copper ($\sim 10^9$ A/cm^2) and the thermal conductivity of an isolated carbon nanotube lies in the range 1750–5800 W/mK [4]. This unique electronic behavior allows the use of nanotubes in the development of electronic components (transistors and capacitors), gas sensors and electron emitters in cold light lamps.

Another peculiar property of CNTs is their mechanical strength. CNTs have extreme tensile strength ($\sim$30 times stronger than carbon fiber) and, unlike carbon fiber, which easily fractures under compression, CNTs may assume a conformation similar to a twisted rope, elastically relaxing when the stress is removed (elastic modulus of $\sim$1TPa) [5]. Besides stretching and compressing, nanotubes can be twisted and folded without breaking. Molecular dynamic simulations confirm that in many cases when stress is removed, the nanotubes can reassume their original shape [6].

Finally, the biocompatibility and non-cytotoxicity of functionalized CNTs allows their use in biological applications [7–9]. Carbon nanotubes can therefore be viewed as exceptionally flexible and resilient heat- and charge-conducting "needles". They have the ability to interact with or cross biological membranes and deliver biomolecules into the cytoplasm. In addition, their extremely high ratio of surface area to volume confers them the capacity to provide multiple attachment sites for various bioactive molecules and, therefore, to generate a hybrid material of high specificity and selective biological function or improved synergistic properties, which can be used in gene therapy, immunotherapy, tissue regeneration and the diagnosis of different diseases. Figure 8.1 summarizes the CNT applications in the biomedical field.

8.2.2 Synthesis of Carbon Nanotubes

Significant variation in grade of purity, size (length and diameter), chirality distribution, electronic behavior, aggregation, graphitization and surface composition is obtained for SWCNT and MWCNT samples, depending on the method and conditions of the synthesis. Such variation defines the CNT use. For bioapplications, both SWCNT and MWCNT have been used, but, for example, the control of purity, length and aggregation state is a crucial requirement.

Historically, the oldest method of production of carbon nanotubes is the electric arc discharge [10, 11]. In this method, a direct-current arc voltage is applied to two graphite electrodes immersed in an inert gas. The anode reaches a higher temperature than the cathode and carbon atoms evaporate. The evaporated carbon atoms coagulate into carbon nanoparticles in the chamber, including fullerenes and MWCNTs, which are deposited onto the cathode. The growing temperature reaches at least 1700 °C, resulting in carbon nanotubes with few structural defects. When a

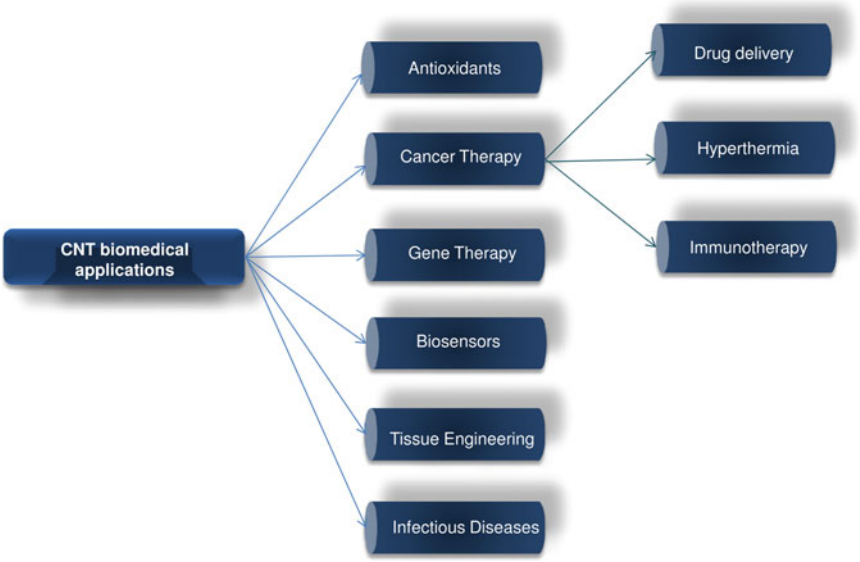

Fig. 8.1 Biomedical applications of carbon nanotubes

graphite anode containing a metal catalyst (Fe or Co) is used with a pure graphite cathode, SWCNTs are generated in the form of soot. The main factors influencing the arc discharge process are carbon vapor concentration, carbon vapor dispersion in the inert gas, temperature, catalyst composition and the presence of promoters and hydrogen. These factors affect the nucleation and growth of nanotubes, their inner and outer diameters, and the type of nanotubes formed [10].

Laser ablation is a method that consists in vaporizing a solid graphite target with a pulsed laser at 1200 °C under continuous He or Ar inert gas flow at a constant pressure of 55 Torr [12]. Nanotubes produced by this method also show good structural quality and purity (up to about 90 % pure) higher than those produced by the arc discharge process. The disadvantage of this method is the small carbon deposit. Laser ablation favors the growth of SWCNTs; MWCNT production requires special reaction conditions [10].

Even though high temperature methods, electric arc discharge and laser ablation produce highly graphitized SWCNT and MWCNT structures, the carbon deposit yield of laser ablation is low and arc discharge produces large amounts of impurities, such as amorphous carbon, fullerenes and catalytic particles.

Currently, these methods have been replaced by low-temperature chemical vapor deposition (CVD) methods. Catalytic chemical vapor deposition (CCVD) is considered an economically viable large-scale process of production of quite pure CNTs, with the advantage of being performed under ambient pressure [10]. In this process, the thermal decomposition of a hydrocarbon vapor, usually methane, ethane, xylene, ethylene or acetylene, is achieved in the presence of a metal catalyst [13]. Nanotubes are produced at temperatures between 700 and 800 °C and grow at

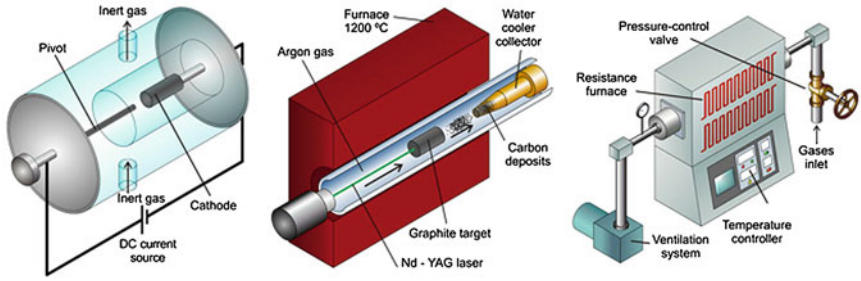

Fig. 8.2 Representative CNT synthesis methods: from *left* to *right*, electric arc discharge, laser ablation (adapted from Ref. [12]) and chemical vapor deposition

the metal catalyst sites. In this process, the carbon precursors are broken apart on the surface of the catalyst particle and the carbon is transported to the edges of the particle, where the nanotubes are formed.

Plasma enhanced chemical vapor deposition (PECVD) is also a suitable method for the synthesis of CNTs. PECVD can be used in several different modes: radiofrequency (RF-PECVD), direct current (DC-PECVD), diffusion (DPECVD) or microwave (MWPECVD). CNTs grow on the catalysts in the reactor and are collected upon the cooling of the system to room temperature. When a liquid hydrocarbon (benzene, alcohol, etc.) is used as a carbon source, the liquid is heated in a flask and an inert gas is purged through it, which in turn carries the hydrocarbon vapor into the reaction zone [14]. Even in the CCVD method, the formation of either SWCNTs or MWCNTs is controlled by the size of the catalyst particle. Figure 8.2 illustrates the methods described here.

The CVD and PECVD methods have been the most used in the last few years. However, the CNT growth mechanisms are still under debate. New CNT synthesis approaches to reach high yields, low costs, high purity, low carbonaceous and metallic impurity contents, narrow length and diameter distribution and suitable electronic properties remain one of the biggest challenges to nanoscientists at present.

8.3 Carbon Nanotube Purification for Bioapplications

As-synthesized CNTs naturally contain carbonaceous impurities, transition metals and ceramic residues from catalysts [15, 16]. Knowing the purity of a carbon nanotube sample for use is a major issue in carbon nanotube bioapplications. Metal catalyst residues and nanotube surface composition (presence of carbonaceous debris, inorganic impurities and defects) may be crucial to define the efficacy and toxicity of carbon nanotubes (CNTs) in biological and environmental systems.

After over two decades of research and development, it is possible to obtain a great variety of SWCNTs and MWCNTs with different grades of purity in the

international market. In general, samples are sold with carbon contents in the range of 70–90 wt% for SWCNTs and <95 wt% for MWCNTs. These ranges do not always express the actual carbon content in the form of CNTs. Many times the samples present large amounts of non-tubular nanocarbons, such as graphitic nano-onions, bare or filled with metals or other carbon nanostructures. Considering that the applications of commercial samples such as in nanomedicine and other bioapplications are still unviable due to their impurity content, both researchers and industries have made a continuous effort to improve the purity and quality of CNT samples [17–19]. The challenge in this field is to efficiently remove synthesis byproducts and preserve the CNT structure and length without introducing new impurities. Due to the similar chemical properties of CNTs and contaminants of carbonaceous origin, the separation methods end up also removing a large part of the CNTs or damaging them, thus compromising the process yield.

The main methods of purification of CNT samples are briefly described in the following sections. We will first deal with processes of removal of the coarser impurities. These processes are usually employed by companies that sell CNTs. Greater attention will be paid to secondary processes of purification, many of which are essential when the goal is CNTs for bioapplications and investigation. The main impurities present in CNT samples commonly used in biostudies will be described in detail and some methods of removal proposed in the literature will be presented.

8.3.1 Primary (Gross) Purification Steps

A large number of purification protocols have been developed after large efforts by scholars and industries over the last two decades, which exploit the chemical and physical differences between CNTs and their synthesis byproducts. Sample complexity requires the use of multiple purification procedures, which many times result in low product yield and considerably increase the cost of the purified material [20, 21]. Additionally, it is not yet possible to define a general protocol, since each synthesis method produces a different set of impurities [16].

In general, the purification protocols combine steps that involve the following types of chemical and physical treatments:

1. gas phase selective oxidation,
2. liquid phase oxidation,
3. physical separation processes (filtration, centrifugation and chromatography).

For most samples, a combination of an initial gas phase oxidation for the removal of amorphous carbon and an extraction step with nitric or hydrochloric acids for the removal of the ceramic support and of free metal particles shows to be efficient enough to produce samples with an adequate grade of purity, for many applications. Nevertheless, this purification route does not achieve the elimination of graphitic particles that are more resistant to oxidation than amorphous carbon or metal particles located inside the nanotubes or encapsulated by carbon shells.

We will briefly approach the main purification procedures that became well-established over time. For further detailed information, the reader may consult reviews on CNT purification available in the literature [15, 16, 19, 22–24].

As previously mentioned, secondary purification procedures to be used with applications that require greater purity are discussed in Sect. 8.3.2.

8.3.1.1 Gas Phase Selective Oxidation

Gas phase selective oxidation is used as an initial step for the removal of amorphous carbon and poorly graphitized carbon nanomaterials with a decomposition temperature lower than that of CNTs. Different atmospheres are proposed in the literature, such as oxygen/air atmosphere [25–28], dry [29] and moist O_2-air mixtures [30], moist air [31], carbon dioxide (CO_2) [32, 33], hydrogen (H_2) [34, 35], ammonia (NH_3) [36, 37] and chlorine (Cl_2) [35, 38, 39]. The treatment temperatures depend on the types of samples and atmospheres and may range from 250 to 1000 °C. In general, the treatments are carried out at a constant temperature (isothermal or static condition); however, some authors support dynamic oxidation treatment [40]. Special attention is required in the purification of SWCNT by this method, since they are less resistant to oxidation than MWCNT and even some impurities such as multi-shell graphitic particles [35].

8.3.1.2 Liquid Phase Oxidation

Due to efficient and easy industrial scaling-up, liquid phase purification certainly is the most used procedure in the purification of CNT samples. In this procedure, different agents are used to oxidize amorphous carbon, metal residues and ceramic supports selectively. Among the most commonly used oxidants are nitric acid (HNO_3) [41–43], hydrochloric acid (HCl) [44, 45], hydrogen peroxide (H_2O_2) [46–48] and potassium permanganate ($KMnO_4$) [49]. Their mixtures can also be used, such as HNO_3/HCL and H_2SO_4/HNO_3. In liquid phase oxidation, the sample is generally heated under reflux with an oxidizing agent aqueous solution; however, ultrasonic- [25, 44, 50, 51] and microwave- [52, 53] assisted digestion have also been largely used.

8.3.1.3 Physical Separation Processes

Due to the chemical similarity between tubular carbon and some carbonaceous impurities, in many cases greater purification can only be achieved with a combination of chemical and physical separation processes. The latter are based on differences in the physical properties (e.g. morphology, density, size and aspect ratio) of CNTs and the impurities. Usually the samples are dispersed in solvents or

surfactant aqueous solutions by ultrasonication [54–56] before separation. The most common physical separation methods are microfiltration [57, 58], centrifugation [59, 60], size exclusion chromatography [61, 62], gel permeation chromatography [63], magnetic separation and ultrasonication [54–56].

8.3.2 Secondary (Fine) Purification Steps

The previously discussed purification steps enable the production of CNT free of most impurities in typical yields from 89 to 95 wt%. Such samples have been successfully used in many academic and technological applications. However, remaining impurities in the commercial sample still are an obstacle to their use in some areas, such as in biological [64–66] and electrochemical [67, 68] applications and in applications involving magnetic properties [69, 70]. In these cases, the presence of impurities such as encapsulated metals, graphitic onions and carbonaceous debris may make the expected CNT performance unattainable or the understanding of their behavior difficult. As a result, additional fine purification procedures and extensive and careful characterization of the composition of the samples are required.

Concerning bioapplications, two problems arise regarding the purification of CNT using strong acids: (1) the introduction of defects, which is followed by the partial functionalization of carboxyl, aldehyde, alcohol, phenol and other oxygenated groups [25] and (2) the generation of oxidation debris around CNT surfaces [71]. Defective and chemically modified CNT will behave differently in vitro and in vivo from high-purity crystalline CNTs [72]. Thus, the extension of the chemical modifications produced by the purification protocol is extremely important in biological studies and must be carefully assessed and published. Manufactures rarely provide information on the actual state of the surface of a CNT sample and it has to be evaluated by the user.

Secondary purification of samples previously purified by manufactures are often necessary to remove residual impurities. Some of the protocols proposed in the literature for the removal of carbonaceous debris, defects and functional groups and bare and metal-filled graphitic nanoparticles are briefly described below.

8.3.2.1 Removal of Oxidation Debris

Before starting a study with any commercially available samples, it is essential to assess the chemical state of the nanotube surface for the presence of defects, functional groups and carbonaceous impurities. This evaluation has to be performed with combined techniques. The most commonly used are Raman spectroscopy, Fourier transform infrared spectroscopy (FTIR), thermogravimetric analysis (TGA), potentiometric titration, X-ray photoelectron spectroscopy (XPS) and high-resolution

transmission electron microscopy (HRTEM) [73]. In most pre-purified samples, the CNTs are covered by carbonaceous debris, as shown in Fig. 8.3 (left). Such debris are polyaromatic fragments resulting from the rupture of the graphitic sheet by acid attack to existing wall and tip defects or the oxidization of carbonaceous impurities, such as amorphous carbon, present on the surface of as-grown CNTs. Due to the structural similarity, such debris cling to the CNT surface via π-stacking [74], as shown in Fig. 8.3 (right).

These polyaromatic fragments have various functionalizations such as carboxylic (–COOH), carbonyl (–C = O) and hydroxyl (–COH) groups [25, 75] and were initially associated with humic and fulvic acids [76]. More recent studies, however, have demonstrated the chemical differences between oxidation debris and natural organic matter compounds [77]. Because they are highly functionalized and have great affinity to the graphitic sheet, they act as excellent surfactants to CNTs.

Special attention must be paid to the removal of oxidation fragments in carboxylic samples, either commercial or prepared in the laboratory as the treatment with the carboxylation process acids is even more aggressive. Most processes are based on CNT reflux or sonication in strong acid mixtures such as H_2SO_4 and HNO_3 [43]. The high dispersability of samples thus carboxylated has been demonstrated experimentally, which is due mainly to the presence of these fragments where most functional groups introduced by the acid treatment are concentrated [74, 76]. Nevertheless, many research groups have demonstrated that the functionalization of CNTs via carboxyl group (–COOH) reaction still is possible, even after the removal of the fragments [78, 79].

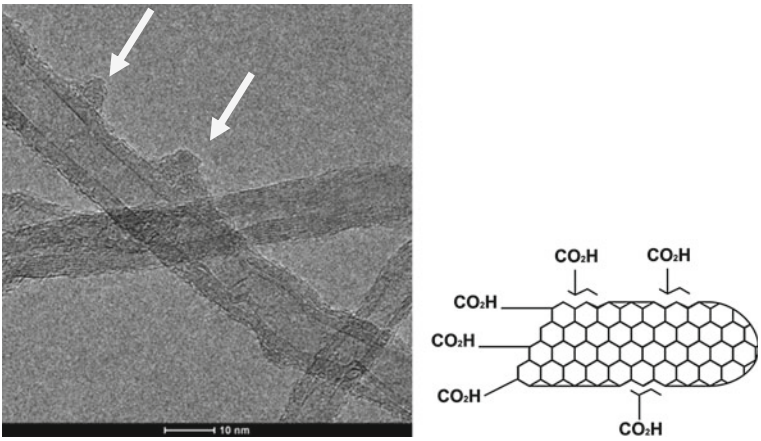

Fig. 8.3 (*Left*) HRTEM image of a purified commercial MWNT material showing the CNT randomly covered with carbonaceous debris. Large build-ups can be seen in some regions (*arrows* in the image); (*right*) schematic illustration of a SWCNT stabilized by carboxylic acid containing polyaromatic fragments

Regarding the toxicity, the experts in the field point out the need for purification and extensive characterization of CNTs so that the results are reliable and comparable [72].

The presence of carbonaceous impurities after nitric acid treatment has long been documented both in the purification of SWCNTs [25, 80] and MWCNTs [81]. Some procedures for the removal of carbonaceous fragments have been suggested and evaluated [73, 76, 82–90]. They involve the solubilization of the fragments by prolonged digestion in a NaOH solution followed by fragment separation by filtration, centrifugation, ultracentrifugation or a combination of these methods. Dr. Silvia Giordani's group has contributed significantly in this area. They have recently systematically reevaluated the acid treatment routes for the introduction of oxygenated functional groups into CNTs and proposed improvement to the protocols for the removal of carbonaceous fragments [85–87, 89]. Wu and Mitra [90] have also recently proposed the use of microwave induced reactive base wash (MRW) for the removal of oxidation debris. They reported that the procedure saves both on time and reagents in comparison to the conventional method based on prolonged reflux.

Recent results by our group have revealed the importance of a final washing step with DMF [73] following the steps in the protocol for the removal of oxidation debris from MWCNT materials described below:

1. 100 mg of MWCNT sample are dispersed in 100 mL of 2 M NaOH by horn sonication for 5 min and refluxed overnight under nitrogen atmosphere. Generally, an orange brown solution is obtained in this step.
2. After filtering the mixture through a 0.45 μm PTFE membrane, and wash neutralization with deionized water, the sample is washed with 100 mL of 2 M NaOH and 50 mL of 1 M HCl neutralization with deionized water is performed after each wash.
3. Next, the samples are washed with ethanol and dried in air at 80 °C overnight.
4. Sample dispersion in 2 M NaOH is achieved by bath sonication for 20 min (135 W), after which the sample is neutralized and washed as previously described.
5. The sample is then dispersed in *N,N*-dimethylformamide (DMF) by bath sonication for 1 h (135 W), filtered through a 0.45 μm PTFE membrane and vacuum dried at 150 °C for 3 days.

8.3.2.2 Removal of Carbon-Coated Metal and Graphitic Onion Nanoparticles

Another important aspect to consider in biological studies with CNTs is the remaining metal content found in CNT samples purified by the classical methods. Part of the metal used in the synthesis remains in the material in the form of nanoparticles coated with mono- and multilayered graphitic sheets (nano-onions) or within nanotube cavities (see Fig. 8.4). There is a debate in the literature on the role

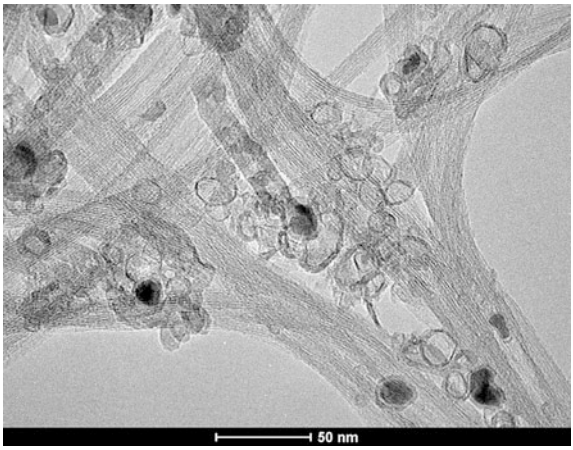

Fig. 8.4 Low-magnification TEM image of a commercial purified SWCNT sample prepared by electric arc discharge. The material contains many graphitic onions, empty or filled with metallic nanoparticles

of metallic impurities in the electrocatalytic properties of CNTs [64, 67, 68, 91]. Pumera et al. reported that amounts as little as 0.01 wt% of metallic impurities are sufficient to determine the electrochemical behavior of CNTs [67, 91]. These results have raised discussion on the role that metallic impurities play into toxicity of CNTs and their interaction with biological media [72]. Metallic residues, especially Fe nanoparticles, have been pointed out as being responsible for the toxic effects observed in certain CNT samples [65, 66]. Professor Robert Hurt's and Professor Agnes Kane's research groups at Brown University have called attention to sources of metals present in CNTs which may become available during manipulation: (1) metallic nanoparticles encapsulated by defective graphitic coatings and (2) purification byproducts, such as metallic salts, that remain deposited on the surface of nanotubes [65]. They have proposed a protocol for the elimination of bioavailable metal in pre-purified samples based on a treatment with a non-oxidizing strong acid solution (3 M HCl) under ultrasonication and incubation at room temperature for 24 h, followed by multiple hot water washing steps. After this purification step, the remaining metal is supposed to be stable in physiological medium, and, therefore, not bioavailable, for two months. A recent study demonstrated that metal residues apparently protected by carbon were released to the reaction medium during dispersion by water-bath sonication [92]. The metallic impurities inhibited the proliferation of neural stem cells.

Special attention has been paid to the removal of Fe impurities in SWCNTs synthesized by the HiPCO method [93–95]. The manufacturer's purified material has a high metallic impurity contents, ~17 wt% of residual material by TGA [79]. Charron and collaborators performed a detailed study of the removal of these impurities and found two types of particles: Fe particles with diameters in the order of 3–6 nm and 10–20 nm encapsulated in graphitic shells of various thicknesses, and various Fe_3C nanoparticles grouped in bunch shape with diameters from 10 to 20 nm and encapsulated in graphitic layers 2–3 nm thick [93]. The treatment of these Fe-based nanoparticles by a multistep purification procedure with gas phase

oxidation and HCl treatment was inadequate to remove all the impurities and resulted in great material losses (up to 85 %). Recently, Ghosh and coworkers proposed a purification method for the removal of residual metallic catalyst in HiPCO SWCNTs based on magnetic purification [95]. This process uses permanent magnets to remove carbon-coated metal nanoparticles and SWCNT aggregates from surfactant-assisted aqueous dispersions.

The removal of empty and metal-filled nano-onions is particularly important for SWCNT prepared by the arc discharge method. In these cases, impurities may correspond to 30 % of the starting materials [96]. The efforts to remove these carbon nanoparticles have been based on a combination of chemical and physical methods, such as material dispersion using surfactants [54], or acid treatments [97], followed by centrifugation [56, 98] and magnetic separation steps [97, 99–101].

8.4 Carbon Nanotube Functionalization for Bioapplications

The nano/bio interface interactions between CNTs and biomolecules to form hybrid functional assemblies are an innovative and promising nanobiotechnology research area. However, the highly hydrophobic surface, the capacity to form insoluble aggregates in aqueous solutions and the cytotoxic effects of CNTs are still current challenges to be overcome in order to promote such interactions. For that, two main strategies have been widely explored: covalent and non-covalent CNT functionalization. Although different routes of CNT surface chemical modification are reported in several reviews [102–104], we highlight here three approaches that have been broadly explored for biomedical applications.

8.4.1 Carbon Nanotube Oxidation and Further Amidation Reactions

Oxidation was the first covalent functionalization method proposed for SWCNT. In a remarkable work reported by Richard Smalley's group, raw (pristine) carbon nanotubes were refluxed in strong oxidizing agents, such as nitric acid (HNO_3) associated or not with sulfuric acid (H_2SO_4). The process results in short and open tubes and wall defects bearing oxygenated groups, particularly carboxylic acids (Fig. 8.5a) [42, 43, 105]. As the CNT ends are more reactive than their sidewalls, a variety of oxygenated functional groups have been grafted mostly at the nanotube tips and in the intrinsic defects on the sidewalls [106]. Since 1998, an infinity of oxidation routes have been reported aiming mainly to reduce the use of toxic reactants and attain a more controlled stoichiometry and position of chemical groups with concomitant decrease in defect density. Several protocols based on different

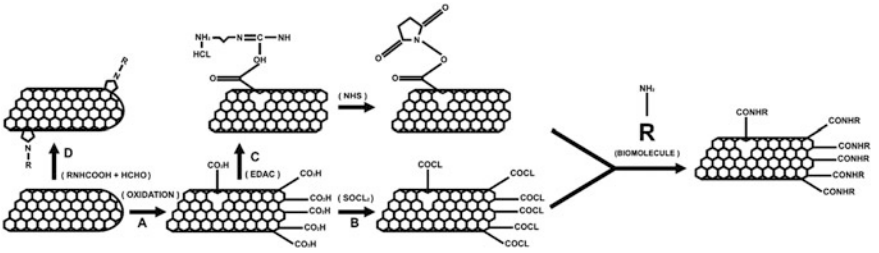

Fig. 8.5 Covalent functionalization of carbon nanotubes **a** oxidation highlighting the introduction of carboxylic acid groups, **b** amidation reaction via acylation with $SOCl_2$, **c** amidation reaction via carbodiimide, and **d** 1,3-dipolar cycloaddition (Prato reaction)

oxidizing agents, such as H_2SO_4, H_2O_2, H_2SO_4 + H_2O_2, HNO_3, NaClO, HCl, $SOCL_2$, K_2MnO_4, O_3 and air have been reported [107–109]. Acid (–COOH) or basic (–OH) sites can be formed on the CNT surface depending on the nature of the oxidizing agent and the experimental conditions. Generally, gas phase oxidation results in a higher concentration of basic groups, while liquid phase oxidation produces mainly acid sites, for similar reaction conditions [108]. With regard to acid digestion, the density of acid or basic functional groups on the CNT surface is highly dependent on the initial condition and less dependent on the nature of the oxidizing agent [109]. Besides the differences between nanotube surfaces regarding the several oxidation routes, extensive purification protocols needed for CNT bioapplication (as discussed in Sect. 8.2) also may completely change the CNT surface chemical composition. In a recent work, for example, Pacheco and coworkers studied the actual surface characteristics of oxidized MWCNT synthesized by three common oxidation routes: microwave-assisted acid oxidation, hydrogen peroxide reflux, and Fenton reaction, before and after purification. Surface composition differences (hydroxyl/carbonyl relative presence) were quali- and quantitavely characterized not only due to oxidation method but also due to time of reaction. They reported a reduction of almost 50 % in the total oxidized group concentration in the samples after purification, showing the importance of this step to reveal the actual chemical composition of the CNT surface [73]. The importance of all these routes is that oxidized CNTs show improved biocompatibility and dispersibility in water and biological fluids, which may lead to further important derivatizations. Following the latter approach, carboxylic acids, which are relatively inactive under ambient conditions, can be chemically activated by highly reactive agents, such as thionyl chloride ($SOCl_2$) (Fig. 8.5b), oxalic chloride (COCl2) or carbodiimide (EDC or DCC) (Fig. 8.5c), allowing the attachment of a wide variety of biomolecules to the CNT surface by stable covalent bonding. The reactive intermediate groups obtained after activation condense with aliphatic and aromatic amine or alcohol and produce amide (–NHR) and ester (–HOR) derivatives [110–113].

The amidation reaction of CNT-COOH through acylation with $SOCl_2$ was the first reported covalent functionalization strategy. It allows the solubilization of SWCNTs in organic solvents [106]. Using the same approach, but focusing on

biological and biomedical applications, Wang et al. [114] performed the covalent amidation of SWCNTs with various amine compounds and two enzymes (porcine pancreas lipase and amino lipase). However, amidation via carbodiimides, using for example 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC), has been chosen as a route over the acyl chloride intermediate formation to attach a variety of biomolecules onto the carbon nanotube surface. EDC is soluble in aqueous media (water, buffer solution and culture medium), which is an important feature when working with biomolecules, and the covalent reaction can be performed at room temperature [115, 116]. In addition, it is more stable and resistant to the hydrolysis of direct activation products (*O*-acylisourea) and it is not corrosive. Amidation via carbodiimide is usually carried out in two-steps: EDC first reacts with a carboxyl group, forming an amine-reactive *O*-acylisourea intermediate, which subsequently reacts with an amine group to produce a stable amide bond. To solve the instability of the *O*-acylisourea intermediate, N-hydroxysuccinimide (NHS) or Sulfo-NHS can be subsequently used to stabilize the intermediate by converting it to a semi stable amine-reactive NHS ester [112, 115, 116] (Fig. 8.5c).

Xu et al. [117] investigated fluorescent visualization of functionalized CNTs in aqueous solution using anti-IgG-Cy5 immobilized CNTs via a two-step process of diimide-activated amidation. CNTs functionalized with fluorescent labeled protein were highly compatible and showed stable fluorescent emission [117]. Liao and Zhang [118] designed a novel biomaterial with potential for photodynamic and photothermal cancer therapy, exploring 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide covalent route to link carbon nanotubes modified with chitosan (CS) to phycocyanin (PC) (MWNT–CS–PC). Cytotoxicity experiments showed that MWNT–CS–PC inhibited cell growth. Moreover, with irradiation with NIR light (808 nm) or visible light (532 nm), the photo-induced cytotoxicity was indeed enhanced, suggesting that MWNT–CS–PC may potentially serve as a future photodynamic and photothermal therapy against cancer [118]. Covalent binding between the amino-modified plasmid DNA and carboxylated MWCNTs was recently achieved, considering the interest of the delivery of linear DNA fragments. Carboxyl groups were first introduced onto MWCNTs by oxidative treatment, then, the carboxyl groups were activated by 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) to further amidation reaction. The resulting bioconjugate was successfully transformed into chemically competent *Escherichia coli* cells, without the need of a heat-shock step at 42 °C [119].

8.4.2 1,3-Dipolar Cycloaddition (Prato Reaction)

Because of the highly aggressive conditions of functionalization of CNT sidewalls, most functionalization routes may not be desirable for the direct attachment of biomolecules. The addition of reactive functional groups that may be further derivatized is a useful alternative. For example, Prato and coworkers used 1,3-dipolar cycloaddition to functionalize CNTs with pendant amino groups, which

are soluble in water and suitable for several further derivatizations via classical chemical routes [120, 121]. They also demonstrated that oxidation/amidation followed by a cycloaddition reaction permits the generation of doubly functionalized CNTs. The 1,3-dipolar cycloaddition of azomethine ylides, named Prato reaction, is achieved by condensation of an R-amino acid and an aldehyde to create pyrrolidine rings [122, 123] (Fig. 8.5d). Since these reactions are compatible with aqueous and biological media, this approach has opened new avenues for CNT bioapplications. After the Prato reaction was first described for biological purposes [124], several collaborators and other groups have explored it to covalently interact an infinity of targeting ligands and/or therapeutic agents. O'Connell and coworkers have demonstrated efficient cell membrane internalization by methotrexate (MTX)—functionalized CNTs via 1,3-dipolar cycloaddition [125]. Kostarelos and coworkers (2002) have assessed the in vivo anti-tumor activity of a CNT-based siRNA delivery system in mice bearing human lung carcinoma. 1,3-dipolar cycloaddition covalently functionalized MWCNTs via terminal amino groups were used as a carrier of a pro-apoptotic siRNA sequence (siTOX). The siTOX-MWCNT complex significantly inhibited tumor growth and prolonged animal survival when compared to controls (MWCNT with a non-coding sequence (SiNEG), functionalized MWCNT alone, and both siTOX and siNEG delivered with cationic liposomes) [126]. Additionally, Bianco and coworkers (2005) demonstrated for the first time the potential of CNTs to present bioactive epitopes in an appropriate conformation both in vitro and in vivo to the immune system. B cell epitope from the foot-and-mouth disease virus (FMDV) was covalently linked to mono- and bis-derivatized CNTs by 1,3-dipolar cycloaddition [127]. The immunization of mice with FMDV peptide-CNT conjugates elicited high antibody responses as compared to free peptides [128]. The same group further studied a different delivery approach of therapeutic molecules. A strategy for orthogonal multiple functionalization in which amphotericin B (AmB) and fluorescein were 1,3-dipolar cycloaddition covalently bound to MWCNT was explored. The AmB-MWCNT complex was easily internalized into mammalian cells without any specific cytotoxic effect. Furthermore, attachment to MWCNT preserved the AmB high antifungal activity against three species of pathogenic fungi [129].

8.4.3 Non-Covalent Functionalization

Despite the high solubility and low cytotoxicity of covalently functionalized CNTs, they are associated with a change in the CNT sidewall carbon hybridization from sp^2 to sp^3 and thus at least a partial loss of conjugation or creation of defects upon reaction. In turn, non-covalent functionalization of nanotubes with amphiphilic surfactant molecules, polymers and biomolecules [130, 131] offers the advantage of conserving the nanotube electronic structures by preventing the rupture of the nanotube sp^2-conjugated structure. Many research groups have explored this approach to raise the solubility of pristine CNTs in water, reduce their hydrophobicity, decrease

their aggregation and size polydispersity and consequently diminish their toxicity. The non-covalent interactions occur via van der Waals forces, including π-π interactions [132]. Even though these interactions provide a relatively weak force between the functional group and the CNTs, the collective strength of the set of interactions is larger than the sum of the individual interaction strengths and can give rise to interesting cooperative effects.

For different biological purposes, biomolecules can be adsorbed on the CNT surface directly or indirectly. In the last case, the biomolecule-CNT attachment is promoted through covalent or non-covalent interactions with an intermediate molecule, such as polymers. Polyethylene glycols (PEGs) are one of the most effective classes of polymers used as dispersing agents due to their hydrophilic, neutral and flexible nature. These characteristics give the nanoparticle surface great resistance to opsonization, which is fundamental in the triggering of the recognition of foreign particles and microorganisms by mononuclear phagocyte system cells (SFM) [133, 134]. Furthermore, PEG is a non-toxic and non-immunogenic compound, it is highly soluble in water and has been approved for use by the US Food and Drug Administration.

Exploring the indirect approach, Hongjie Dai and coworkers constructed a complex to cancer therapy based on CNTs non-covalently functionalized with a phospholipid (PL) conjugated to polyethylene glycol (PEG-PL). Two chains of phospholipid fatty acids were linked with the nanotube surface by hydrophobic interaction, leaving free PEG ramifications for further derivatization [135–141]. A dispersion of highly individualized tubes was obtained. Such functionalization was able to increase the longevity of nanotubes in the systemic circulation due to steric hindrance of the long PEG chains during the nanotube-macrophage interaction. The increase in the hybrid residence time in the system made the CNTs more susceptible to reach the target sites. Thereafter, Dai's group investigated the potential of CNTs functionalized with PEG-PL as drug carrier systems for the treatment of cancer [67, 71]. Kam and coworkers tested a SWCNT functionalized with PEG containing folic acid on one edge and a hydroxyl group on the other edge covalently bound to a phospholipid. Folic acid was used to allow selective binding to folate receptors, which are expressed by cancerous cells. The specific killing of cancer cells and the preservation of healthy cells were demonstrated [138, 142].

Similarly to Dai's approach, several small anticancer drug molecules non-covalently attached to CNT surface were also carried to tumor cells. Doxorubicin (DOX), a well-known chemotherapeutic agent used in the treatment of many human cancers, has been loaded onto the surface of PEGylated SWNTs by π-π stacking [143]. The PEG-coated SWNTs-DOX complex injected in an animal model of breast cancer had enhanced therapeutic efficacy and markedly reduced toxicity when compared to free DOX and a liposomal formulation containing doxorubicin (DOXIL) [143]. The same group further studied the behavior of a PEG-coated SWNTs-DOX complex in SCID mice bearing Raji lymphoma xenografts. A higher uptake by the tumor cells and a greater inhibition of tumor growth were observed when compared to free DOX [140]. Simultaneously to this study, Ali-Boucetta et al. [144] showed the ability of DOX to also interact non-covalently

with MWCNT and the enhanced capability of the MWCNT-DOX supramolecular complex to kill in vitro MCF7 human breast cancer cells. In a different approach, specific targeting ligand-receptor interaction has been explored to further enhance the retention of macromolecules in the tumor site and the therapeutic efficacy [144]. Liu et al. [145] studied the DOX delivery capability in mice bearing human glioblastoma cancer cells using a targeting agent named cyclic arginine-glycine-aspartic acid (RGD) peptide, which acts as a recognition motif for integrin $\alpha_v\beta$ receptors, overexpressed in a wide range of solid tumors. The PEG-coated SWCNT-DOX complex bound to a targeting agent and tested on integrin $\alpha_v\beta_3$- positive cells showed enhanced drug delivery when compared to the complex without RGD [145]. In another study conducted by the same group, paclitaxel (PTX) was conjugated to branched PEG-coated SWCNTs via a cleavable ester bond [138]. Paclitaxel is a poor water-solubility anticancer molecule known commercially as Taxol. Its circulation time is very short and the product (Cremophor EL) used to solubilize the drug is itself toxic. However, the PTX-SWCNT conjugate showed a higher efficacy in suppressing tumor growth than clinical Taxol in breast-cancer-bearing mice, as well as prolonged blood stability and decreased toxicity [138]. Non-covalent conjugation of anti-cancer cisplatin to PL-PEG-SWCNT has also been studied [146].

PEGylation is, therefore, a suitable strategy to create new multifunctional nanobiological systems. PEG coating increases nanoparticle circulation time in the blood, allowing the use of nanoparticles in biomedicine without adverse body reaction, such as oxidative stress and inflammatory processes. However, one problem is the polymer excretion from the body. PEGs are normally excreted through the urine or feces and may accumulate in the liver [147].

Our research group has contributed to the development of a new approach for siRNA cellular delivery using siRNA attached non-covalently to carboxyl-functionalized SWCNTs. The SWCNT–siRNA delivery system was transfected effectively in both primary cell and hard-to-transfect cell lines, such as neuronal cells and cardiomyocytes [148]. Following the same functionalization approach, carboxyl-functionalized MWCNT complexed with DNA promoted gene delivery to Nile tilapia spermatogonial stem cells with higher transfection efficiency than cationic lipids and electroporation, also causing less cell death [149]. More recently, Faria and coworkers developed a vaccine formulation comprising MWCNTs as a delivery system for Cancer Testis Antigens NY-ESO-1, allied to CpG oligonucleotides (CPG-ODNs) as immunological adjuvants. Both biomolecules were non-covalently attached to shortened oxidized MWCNTs. After showing the efficiency of the complex internalization into dendritic cells in vitro and in vivo, the hybrid NY-ESO-1-MWCNT-CPG-ODNs was tested as an anticancer vaccine formulation. Vaccination significantly delayed tumor development and prolonged mouse survival, showing strong prophylactic and therapeutic protection against cancer. Following the current challenges in understanding the influence of the physicochemical characteristics (surface chemistry and size) of nanostructures and the nano-bio interface on the synergistic effect of the hybrid formation on its bio-response, MWCNT-ovalbumin (OVA)-CPG hybrids were also tested as

delivery systems and fully characterized. Short oxidized MWCNTs containing two functional groups in every 100 carbon atoms, mostly carboxylic acids (0.8 mmol. g^{-1}) and phenols (0.9 mmol. g^{-1}) distributed at the ends and wall of the tubes, were densely coated by biomolecules. A narrow length distribution with 76 % of the tubes below 600 nm (48 % below 250 nm) was statistically measured. Figure 8.6 illustrates the general aspect of the shortened MWCNT dispersed in the vaccine formulation (left) and the assembly of patterned OVA entanglements adsorbed on a first layer of protein covering the surface (right). The authors suggest a predominant electrostatic character for the interaction between oxidized MWCNT and OVA, while the oxidized MWCNT-CPG interaction strength is provided predominantly by π-stacking [150].

Despite their immense potential benefits in therapy and prophylaxis, the toxicity of CNTs is a yet little investigated major challenge. Several studies have shown that the increased solubility of functionalized CNTs results in low toxicity. By comparing CNTs dispersed through functionalization and assisted with surfactant, functionalized CNTs showed low cytotoxicity, whereas surfactant-dispersed CNTs showed lower toxicity than pristine CNTs [151]. Dumortier et al. [152] in a study conducted on immune system cells using two different functionalized CNTs, one through 1,3-dipolar cycloaddition and another through oxidation/amidation, showed that both systems were taken up by B and T lymphocytes, as well as by macrophages in vitro without affecting cell viability. In addition, various reports suggest that once the functionalized CNT releases the drug into a specific area, it is gradually excreted via biliary pathway without causing any significant side effect [153]. Despite great evidence of enhanced biocompatibility, the biological response and cytotoxic effects of CNTs are highly dependent on their surface chemistry and the purification and functionalization processes. Continued investigation to understand the role of physicochemical properties in CNT interactions with biological systems is essential to develop safe nanomaterials with useful therapeutic and imaging properties.

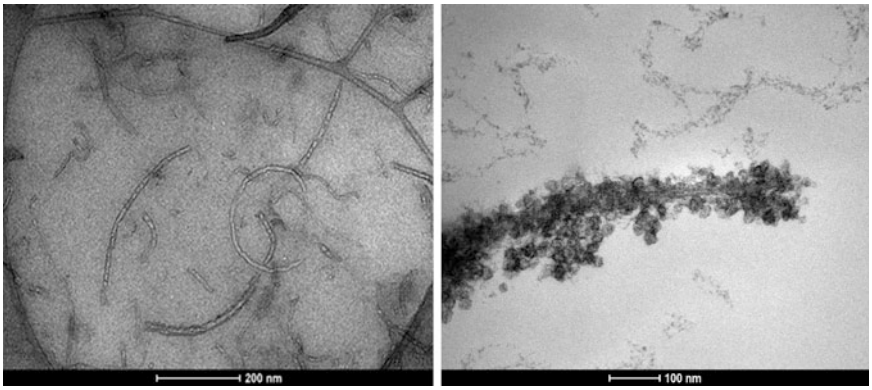

Fig. 8.6 Transmission electronic microscopy images of (*left*) shortened tubes in a MWCNTs-OVA-CpG vaccine formulation and (*right*) the MWCNT-OVA complex showing the patterned assembly of OVA entanglements on an oxidized nanotube surface

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