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Carbon nanotube-mediated delivery of peptides and genes to cells: translating nanobiotechnology to therapeutics

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During the last few years, there has been a tremendous amount of optimism and expectation about nanotechnology and its impact on various fields including medicine and pharmaceutical development. One of the most promising materials being developed during the nanotechnological renaissance we are currently experiencing is the carbon nanotube. Before any biology-related application can even be envisaged, the aqueous solubility of carbon nanotubes has to be resolved. Recently, a variety of methodologies have been proposed which lead to biologically compatible carbon nanotubes. Covalent functionalization of their surface is one methodology, allowing the first attempts towards applications in the field of nanomedicine. The possibility of incorporating functionalized carbon nanotubes into cells and the biological milieu offers numerous advantages for potential applications in biology and pharmacology. One of the most promising is their utilization as a new carrier system for the delivery of therapeutic molecules. In the present article, the first attempts to transform carbon nanotubes from biologically incompatible nanomaterials to biologically relevant components of advanced therapeutics and the ensuing novel structures obtained in our laboratories are presented.

Key words: Carbon nanotubes – Nanomedicine – Peptide delivery – Vaccination – Gene delivery – Gene therapy.

I. INTRODUCTION

Nanomaterials are expected to revolutionize materials science, technology and a wide range of industries. By allowing control of materials' structure on a super-fine scale, this technology realizes improvement in functions and characteristics of materials as well as creation of new functions. Nanomaterials are commonly defined as materials with an average grain size less than 100 nm and include nanoparticulate ceramics, metals, alloys, and semiconductors in the form of dry powders, liquid dispersions, coatings, carbon black powders, fullerenes and nanotubes. Significant enhancement of optical, mechanical, electrical, structural and magnetic properties are commonly found with these materials. In the biomedical field, colloidal nanoparticle systems have been employed for a number of applications from enzyme immobilization to the development of delivery systems for anticancer agents.

1. Nanobiotechnology

The field of nanobiotechnology is currently being defined by the emergence of a variety of nanomaterials or novel combinations of already existing nanocomponents (such as colloidal particles below 100 nm) in order to render them with improved functionalities of biological significance. Nanobiotechnology currently includes a wide variety of technological innovations that are aimed at biological system applications [1]. The delivery of drugs and other therapeutic or diagnostic agents naturally falls within the realm of nanobiotechnology even though the development of advanced, functional delivery systems precedes the emergence of nanobiotechnology as a field. The use of novel nanomaterials such as fullerenes, quantum dots, carbon nanotubes in biologically relevant applications certainly falls within the remits of the nanobiotechnology field [2].

However, the transformation of such nanomaterials into nanobiotechnological components requires significant modifications of their physical and chemical characteristics [3]. Great care should also be exercised in differentiating between speculative opinions or expectations of the impact of novel nanomaterials and the reality of engineering and transforming them to biocompatible tools. Novel nanomaterials will gradually impact biomedicine either as advanced biosensors, diagnostic or drug delivery systems, however, modifications of their physicochemical characteristics have to be developed in tandem with studies determining their biocompatibility, toxicity and biological effectiveness.

2. Carbon nanotubes

Perhaps the most exciting class of nanomaterials are carbon nanotubes (CNT), or "buckytubes" [4, 5]. CNT possess extraordinary properties, including high electrical and thermal conductivity, great strength, rigidity, and are being developed for a wealth of applications, including resilient composite materials, field emission [6], energy storage [7, 8], molecular electronics [9], and many others. After having really attracted the attention of the researchers in the 90s, interest has lied with the synthesis of nanotubes and their physicochemical characterization [10]. Even though great advancements have been made which allow the synthesis of a variety of CNT, major gaps still remain in both the understanding of their basic capabilities and the processing required to produce reproducible CNT batches of identical characteristics and high quality control.

CNT, which consist exclusively of carbon atoms, belong to the family of fullerenes, the third allotropic form of carbon after graphite and diamond. The carbon atoms of the nanotubes are arranged in a series of condensed benzene rings rolled-up into

a tubular form. Carbon nanotubes can be obtained as single-walled (SWNT), characterized by the presence of one layer of cylinder graphene, or multi-walled (MWNT), made-up of several concentric graphene sheets. As the name implies, CNT are object of nanometric dimensions. Most commonly, SWNT have a diameter from 0.4 to 3.0 nm and a length in the range of 20-1000 nm, while the MWNT are bigger objects with a diameter in the range of 1.4-100 nm and a length from 1 to 50 μm. Several methods for the production of both types of tubes and the modulation of their dimensions are available in the literature [11].

CNT have proven difficult to solubilize in aqueous solutions, limiting their use in biological applications [12, 13]. Even though exploration of the biomedical applications of carbon nanotubes is in nascent stages, their utilization is highly anticipated [14, 15]. Although CNT are currently largely exploited in material science, they are also attracting the interest of several research groups in the field of biotechnological, biological and biomedical sciences [12]. The main obstacle in the utilization of CNT in biology and medicinal chemistry is certainly their lack of solubility in most solvents. Once this is overcome, applications of CNT as nanobiotechnological components for the development of new delivery systems can be explored.

3. Water-soluble functionalized carbon nanotubes

CNT are completely insoluble in most organic solvents and aqueous buffers. For any kind of biologically relevant application, whereby incorporation of CNT in physiological conditions will be required, it is essential that their aqueous solubilization be achieved. Two main methodologies have been developed for this to be achieved: i) non covalent functionalization, and ii) covalent functionalization of CNT. In the case of non-covalent functionalization, another macromolecule (a biopolymer such as protein, DNA, or a surfactant) is allowed to interact with the CNT. CNT surfaces have been found to interact with a variety of molecules, which render their water-soluble character onto the CNT. During covalent functionalization of CNT, a water-soluble molecule is covalently bonded to the external nanotube surface by reaction. In this way, the chemical reactivity that CNT have been found to exhibit towards many reagents is taken advantage of to enhance their solubility. The reported methodologies to achieve covalent functionalization of CNT have recently been reviewed [13, 16, 17].

One of the most powerful organic functionalization methodologies, particularly suitable for the preparation of soluble CNT, is the 1,3-dipolar cycloaddition of azomethine ylides [18]. This reaction works efficiently with both SWNT and MWNT. The functionalized CNT (*f*-CNT) that have been synthesized in our laboratories via this method are sufficiently soluble allowing characterization by standard spectroscopic means (i.e., FT-IR and NMR). The amount of functional groups around the tips and sidewalls of the CNT can be quantified using different techniques, such as absorption spectroscopy, calorimetric analyses, etc. All of these techniques have offered very similar values on the degree of functionalization achieved, which is different for SWNT and MWNT [19]. Whereas the former usually showed a loading of functional groups per gram of material between 0.3-0.5 mmol, MWNT carried about 0.5-0.9 mmol/g. The higher loading for MWNT could be attributed to the fact that they are isolated entities and present a larger surface area available during the addition reaction in comparison to the SWNT, which instead form bundles that are difficult to disaggregate.

II. BIOLOGICAL APPLICATIONS OF CARBON NANOTUBES

Carbon nanotube research has reached a maturity stage that offers an adequate understanding of the structural and physicochemical properties and allows the exploration of their use in various applications, including the biological application. Biotechnological applications of CNT are being anticipated in a variety of fields ranging from microfluidics to bioinformatics. Ever since methodologies to improve the aqueous solubility of CNT (also in biological fluids) were developed, transformation of CNT as viable components of therapeutic agents appropriate for clinical use has been feasible. *Table I* describes the different categories of biology-related applications for which CNT have already been explored. The type of CNT and the type of functionalization (if any) are also described.

1. Biosensors

The detection of molecules (DNA, proteins, enzymes, infectious agents) present in diminutive concentrations in biological environments is difficult if not impossible. Ultra-sensitive assays are required to achieve very low detection limits. Carbon nanotubes have been extensively explored in this area due to their physical and electrochemical properties. Most bioassays depend on hybridization or antigen-antibody interactions [22]. Also, CNT are used to immobilize DNA, proteins, enzymes or anions on their surface, in this way acting as bioelectrodes [21-29] responsible for transduction and amplification of events or as catalysts in biochemical reactions [20]. Nanoscale systems based on carbon nanotubes have already shown great improvements in bioelectronic devices, protein analysis and medical diagnostics [20-29].

2. Tissue engineering

Neural and bone implants commonly fail due to glial scar-tissue formation (central nervous system) or fibrous encapsulation (bone) at the tissue-implant interface. This leads to short-term functionality of the implant and can result in poor clinical efficacy [31]. The rough surface of carbon nanotubes at the nanometer scale has been shown to permit sufficient natural bonding between the implant and the surrounding tissue without wound-healing [31]. Moreover, carbon nanotube matrices have offered a range of possibilities for the design of new composites for neural and orthopedic implants combining numerous features. Besides the electric properties of CNT that allow electrical stimulation of cellular growth [30, 34], and their strong mechanical properties needed to ensure implant robustness [33], CNT are biocompatible and non biodegradable material, permitting their use as long-term implants [30]. Very recent studies *in vitro* [30-34,] have explored the possibilities for the use of CNT in tissue regeneration after damage of the spinal cord, brain or bone tissues.

Table I - Carbon nanotubes in biomedical applications.

	CNT	Functionalisation	*In vitro* model	*In vivo* model	Study aim	Ref.
Biomedical sensors	SWNT	Unmodified	n/a	n/a	Role as catalysts in biochemical reactions (PCR)	[20]
	SWNT MWNT	Oxidized and then coupled covalently with DNA	n/a	n/a	CNT for amplifying enzyme-based bioaffinity electrical sensing of proteins and DNA	[21]
	MWNT	GOD enzyme immobilized on the electrode of gold/multi-walled carbon nanotubes	n/a	n/a	Glucose biosensors	[22]
	SWNT	Oxidized (COOH)	n/a	n/a	Bioelectrodes	[23]
	SWNT	Ferrocenoyl-functionalized	n/a	n/a	Glucose biosensors and phosphate anions	[24, 25]
	MWNT	Immobilized on the surface of a glassy carbon electrode by mixing with horse-radish peroxidase (HRP)	n/a	n/a	On-line glucose and lactate biosensors	[26]
	MWNT	Oxidized and covalently bounded to DNA	n/a	n/a	Miniature DNA biosensors	[27]
	MWNT	Carbon nanotube-containing composite with encapsulated enzymes	n/a	n/a	Development of stable biosensors	[28]
	SWNT	Polyethylene oxide-functionalized CNT	n/a	n/a	Highly specific electronic biomolecule detectors	[29]
Tissue engineering	MWNT	Oxidized (COOH) and chemically bounded to PABS and ethylenediamine	Hippocampal neurons	n/a	Neural prosthesis	[30]
	MWNT	Unmodified or coated with the bioactive molecule 4-hydroxynonenal	Embryonic rat-brain neurons	n/a	Substrate for neuronal growth	[31]
	MWNT	Oxidized (COOH)	L292	n/a	3D network substrate for cellular growth	[32]
	Carbon nanofibers/ nanotubes	CNT reinforced polycarbonate urethane composite	Rat astrocyte cells, human osteoblast cells, PC-12 and 3T3	n/a	Neural or orthopaedic prosthetic devices	[33]
	MWNT	Nanocomposite consisting of blends of polylactic acid and CNT	Osteoblasts	n/a	Substrate to expose cells to electrical stimulation	[34]
Ion channel blockers	SWNT	Oxidized (COOH)	CHO	n/a	Block K' channels	[35]
Translocation into cells	SWNT	FITC or peptide from G protein covalently bounded to CNT	3T6, 3T3	n/a	CNT as delivery systems and their cellular uptake	[36]
	SWNT	FITC or biotin-fluorescent streptavidin covalently linked to CNT	HL60	n/a	Cellular internalization of CNT conjugates	[37]
Cytotoxicity	SWNT	Unrefined	HaCaT	n/a	Risk evaluation of the dermal exposure to SWCNT	[38]
	SWNT	Unrefined	n/a	n/a	Risk evaluation of the airborne and dermal exposure to SWNT	[39]
	SWNT	FITC or peptide from G protein covalently bounded to CNT	3T6, 3T3	n/a	Cellular viability	[36]
	SWNT	FITC and biotin-fluorescent streptavidin covalely linked to CNT	HL60	n/a	Cellular viability	[37]
	SWNT	Unrefined	n/a	Crl:CD(SD)I GS BR mice	Evaluation of the acute lung toxicity of intratracheally instilled SWNT	[40]
	SWNT	Unrefined	n/a	B6C3F mice	Evaluation of the pulmonary toxicity after intratracheally instillation of SWNT	[41]
	SWNT	NH_3^+	HeLa	n/a	Cellular viability	[42]
Antigen delivery	SWNT	FMDV peptide covalently linked to CNT	n/a	n/a	Study of the antigenicity of the FMDV peptide-CNT conjugate	[43]
	SWNT	Mono and bis functionalization with FMDV peptide	n/a	BALB/c mice	Study of the immune response of CNT conjugates	[44]
Gene delivery	SWNT MWNT	NH_3^+ covalently linked and plasmid DNA by electrostatic interactions	HeLa CHO	n/a	CNT as delivery system, cellular distribution of CNT conjugates and gene expression	[42]

3. Ion channel blockers

Ion channels play an essential role in cellular communication (cell-cell and cell-environment). Disturbances in the ionic balance generate information for cells, and the cellular response will interfere with the body homeostasis. Controlling the functionality of ion channels can be very useful in several situations when for example an ionic imbalance results in a disease. Several toxins and therapeutic drugs have ion channels as their targets. One study [35] has shown the susceptibility of K- channels responding in a dose-dependent manner to CNT of certain diameters. Further exploration in this area can lead to CNT per se as therapeutic agents.

4. Cytotoxicity studies

The increasing interest in CNT for electronics to biomedical applications, inevitably will lead to the mass production of these materials in all their conformation: single or multi-walled, functionalized or not. A few studies have lately appeared on the toxicity of carbon nanotubes. Concerning handling of unrefined CNT material by CNT production workers, these studies have focused on dermal [38] and pulmonary [39-41] exposure. Current studies on the utilization of *f*-CNT as carriers for drugs, DNA and proteins and their internalization into cells, have produced new information related with the cytotoxicity of carbon nanotubes [36, 37, 42] on the cellular level.

III. CARBON NANOTUBE-BASED DELIVERY SYSTEMS

Recently, new approaches are emerging in the field of drug delivery, mainly due to the significant advances in nanotechnology and nanofabrication [1]. The future generations of drug delivery devices may well include microchip-controlled release drug reservoirs [45], lipid, peptide or silica nanotubes [46] and carbon nanotubes [47].

CNT are thought to hold particular promise as delivery systems for therapeutic agents. Our laboratories have placed particular attention and effort towards development of CNT as components of delivery systems for immunotherapeutic and genetic intervention purposes. The strategies we followed to achieve the transformation of CNT to viable delivery systems were first to render the CNT water soluble by organic functionalization and then either covalently link peptides or electrostatically complex gene-encoding nucleic acids onto the CNT surfaces (*Figure 1*).

1. Interaction of functionalized carbon nanotubes with mammalian cells

In order to assess the capability of *f*-CNT to act as viable delivery systems, their interaction with mammalian (human) cells was examined. The aqueous solubility and cationic surface character of *f*-CNT (*Figure 1*, molecular structure 1) render them as potentially novel delivery vehicles. In this context, we initially demonstrated the capacity of carbon nanotubes to cross the plasma membrane and distribute throughout the cellular compartments. Ammonium functionalized multi-walled carbon nanotubes (MWNT-NH_3^+) were allowed to interact with HeLa cell cultures. Their interaction was monitored by sectioning the cells and examination by transmission electron microscopy (TEM) [42]. After incubation of the cells with *f*-CNT, followed by washing, staining and embedding into a polymer matrix, slices of 90 nm thickness were cut with a diamond microtome knife for imaging under TEM. The tubes were clearly visible throughout the cellular compartments, distributed throughout the cytoplasm and the nucleus. Although the elucidation of the mechanism of entry requires further investigations, we could rule out endocytosis. This is because inhibitors of endosome-mediated translocation such as sodium azide and 2,4-dinitrophenol and the decrease of the incubation temperature to 4°C did not prevent cellular uptake of the different *f*-CNT. In addition, the TEM images revealed tubes crossing the intracellular membranes reaching the nucleus as nano-needles without evident permanent disruption or damage to the membrane or cellular compartment structures (*Figure 2*).

Cell viability following treatment with *f*-CNT is an important issue that was addressed by using flow cytometry to monitor cell survival. Only 50% of the cells died after a 6-h incubation period with 5-10 mg/ml of nanotube solution, a concentration considered excessively high [42]. We also observed that FITC functionalized carbon nanotubes were more toxic than those with the free ammonium group [36].

However, our currently available data and observations of the ensuing interactions between CNT or *f*-CNT and mammalian cells, even though recently reproduced by others using a differently functionalized CNT system [37], make it difficult to propose a general mechanism of CNT cellular uptake. Overall, these results could be considered as extremely promising, although they need to be improved and complemented with studies on *f*-CNT metabolism, distribution and elimination *in vitro* and *in vivo*.

2. Functionalized carbon nanotubes for peptide delivery

The encouraging observations of the interactions between *f*-CNT and mammalian cells, allowed us to further explore their capacity for therapeutic applications. Immunization with synthetic vaccines, based for example on peptides, elicits reduced immune responses compared to natural pathogens. *f*-CNT can

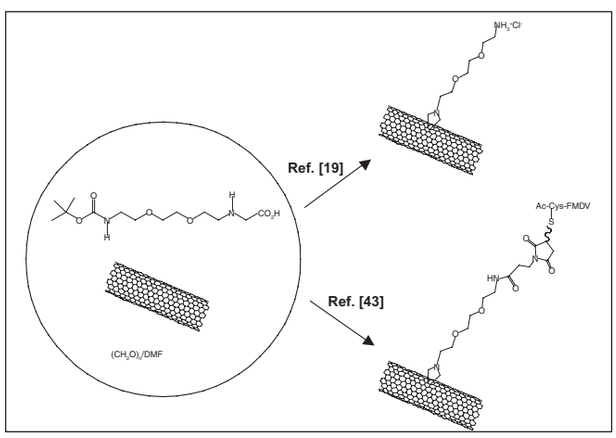

Figure 1 - General approach to the 1,3-dipolar cycloaddition reaction used to functionalize the CNT surfaces. The molecular structures represent the SWNT-NH_3^+ (1) and SWNT-FMDV peptide (2) used for the gene and peptide delivery studies.

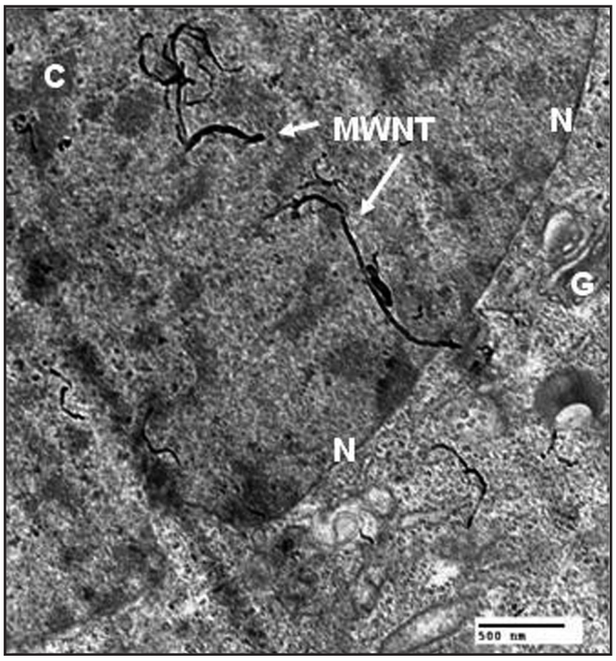

Figure 2 - Ultrathin transverse section (90 nm thick) of HeLa cells treated with MWNT-NH_3^+. C represents chromatin structures in the cell nucleus; N the nuclear membrane; G the Golgi complex; and MWNT and arrows the *f*-CNT. Scale bar is 500 nm.

theoretically be ideal carrier systems for peptide antigens, with a potentially high loading capacity for cargo molecules. In this context, synthetic peptides, considered as potential vaccine candidates, were conjugated to CNT to study their immunological properties (*Figure 1*; molecular structure 2). For this purpose, a model antigen derived from a B cell epitope of the foot-and-mouth disease virus (FMDV) has been coupled to carbon nanotubes [43]. This peptide corresponds to the sequence ^{141}GSGVRGDFGSLAPRVARQL^{159} of the VP1 coat protein of the virus, modified at the N-terminal part with an acetylated cysteine. This peptide was selected because it represents a virus neutralizing and protective epitope. The peptide was linked to the *f*-CNT as a mono- or a bis-conjugate [43, 44], and was found to be presented on the CNT surface in the appropriate conformation for the spatial interaction with the antibody. Immunization studies using the peptide-*f*-CNT in mice clearly enhanced anti-FMDV peptide antibody responses compared to the free peptide and a mixture of the free peptide with *f*-CNT. The antibodies produced were specific to the peptide. Moreover, these studies showed that no antibodies were produced against *f*-CNT. This suggested that carbon nanotubes do not possess intrinsic immunogenicity. In view of these results, *f*-CNT can be considered as an interesting and promising presentation system for the delivery of candidate vaccine antigens based, for example, on peptides and proteins conjugated on the CNT surface.

3. Functionalized carbon nanotubes for gene delivery

The delivery of nucleic acids to target cells represents one of the most important obstacles to successful genetic intervention modalities. Indeed, for successful gene therapy an efficient delivery system is required. This will allow the transfer and expression of the therapeutic gene in the target organ or tissue. Our observations that *f*-CNT can be uptaken and distributed throughout mammalian cells led to their exploration as novel non-viral vectors for the delivery of plasmid DNA. Complex formation between *f*-CNT and plasmid DNA can constitute a novel class of non-viral gene delivery systems.

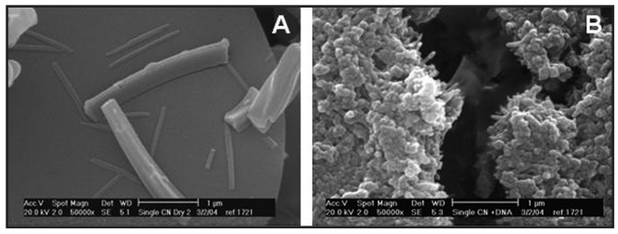

Figure 3 - Scanning electron microscopy (SEM) images of SWNT-NH_3^+ alone (A) and following interaction and complexation with plasmid DNA (B). Scale bar for both images is 1 μm.

Based on the fact that it was possible to exploit the macromolecular and cationic nature of the *f*-CNT to form supramolecular complexes with plasmid DNA, we tested these complexes in gene transfer and gene expression experiments [42]. Other groups have previously studied the interaction of carbon nanotubes with single stranded DNA (ssDNA), aiming to increase the solubility of nanotubes in aqueous solution and to reduce their polydispersity [48]. For the purpose of exploring the potential of *f*-CNTs as gene transfer vectors, we carried out an initial study that focused on assessing the capacity of *f*-CNT to condense plasmid DNA (*Figure 1*; molecular structure 1). We studied an electrostatically formed complex between pCMV-βgal, which would allow expression of the β-galactosidase marker gene, and SWNT-NH_3^+. Interaction between the two components at different charge ratios was carried out and observation of the ensuing supramolecular assemblies using SEM (*Figure 3*) indicated that nanotube-DNA complexes were formed. The *f*-SWNT were present in bundles of different diameters on which the plasmid DNA was condensed by forming toroidal clusters, or globular and supercoiled structures. This observation was extremely encouraging for the subsequent planning of gene delivery and expression experiments. Indeed, we were able to obtain a clear effect by using the DNA complexes with SWNT-NH_3^+ to carry the βgal gene in cells, by monitoring the expression of β-galactosidase. Improved levels of gene expression were obtained for the SWNT-NH_3^+:DNA complex in the range of positive: negative charge ratios from 2:1 to 6:1. Gene expression offered by the complexes between plasmid DNA with *f*-CNT was 5 to 10 times higher than that of DNA alone [42]. These initial gene transfer and expression studies represent the first step towards the exploration of functionalized carbon nanotubes in gene therapy and genetic vaccination applications.

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Carbon nanotubes constitute one of the most intensively researched nanomaterials. Their unique properties offer great

promise for the development of a variety of nanotube-based components in fields ranging from electronics and composites to biomedicine. We are still in the nascent stages of carbon nanotube technology development, particularly in view of biomedically-related applications. The transformation of carbon nanotubes from novel, biologically incompatible materials to useful and effective, yet safe, biotechnological tools requires a lot of effort to improve different nanotube aspects including their efficiency, biosafety, processing and production. Nevertheless, carbon nanotubes have already been explored for a variety of biological applications. The present article describes the proof-of-concept studies of carbon nanotubes as advanced delivery systems for peptides and genes and some of the interesting structures and observations obtained. Further developments of carbon nanotubes as components for therapeutics will certainly follow as a direct consequence of the studies discussed.

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