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Review

Biophilic carbon nanotubes

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ABSTRACT

Carbon nanotubes (CNTs) have been proposed and are actively being explored as innovative multipurpose carriers for biomolecules and diagnostic applications. Their versatile physico-chemical features enable them as a carrier of several pharmaceutically relevant entities and allow them for rational design of novel nanoscale candidates for drug development. Functionalized carbon nanotubes (*f*-CNT) are emerging as a new family of nanovectors for the delivery of different types of therapeutic molecules. The application of CNTs in the field of carrier-mediated delivery has become possible after the recent discovery of their capacity to penetrate into the cells. CNT can be loaded with active molecules by forming stable covalent bonds or supramolecular assemblies based on noncovalent interactions. Once the cargos are carried into various cells, tissues and organs they are able to express their biological function.

In this review, we will describe the potential of *f*-CNT as a vehicle to deliver different types of therapeutic agents into the biological species.

Contents

1. Introduction and background information

In 1991, Iijima reported the first observation of multi-walled carbon nanotubes (MWCNTs or MWNTs) synthesized by arc-discharge method [1]. Shortly afterwards, this discovery was followed by the remarkable announcement of Iijima and Ichihashi having succeeded in synthesizing single-walled carbon nanotubes (SWCNTs or SWNTs) [2]. A major breakthrough occurred in 1996 when Smalley and coworkers at Rice University successfully synthesized bundles of aligned single-wall carbon nanotubes [3], with a small diameter distribution, thereby making it possible to carry out many sensitive experiments relevant to one dimensional (1D) quantum physics. The origin of the exceptional strength of carbon nanotubes can be found in the fact that chemical bonding between carbon atoms inside nanotubes is always of sp^2 type with each atom joined to three neighbours, as in graphite. A variety of superlative mechanical properties are attributed to the strength of the sp^2 carbon–carbon bonds in carbon nanotubes (CNTs) [4]. In addition, a variety of novel electronic properties of CNT's have proven to be a further source of interest in this material. Depending on their diameter and chirality, CNTs are either semiconducting or metallic [5]. Moreover, their nanosized diameters and micro-sized lengths lead to large aspect ratios that make CNTs behave as nearly ideal one dimensional (1D) quantum wires [6]. Indeed, the high mechanical, thermal, and chemical stabilities coupled with their amenability toward structural manipulation have turned carbon nanotubes into technological designer materials of note for use across many platforms.

There are two types of CNTs being reported on. First one is the single-walled carbon nanotube (SWNT) which is composed of a rolled monolayered graphene sheet and the second one is the

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multi-walled carbon nanotube (MWNT) which possesses several graphitic concentric layers. The diameter varies from 0.4 to 2 nm for SWNT and from 1.4 to 100 nm for MWNT, while the length can reach several micrometers for both types of nanotubes. Purification of CNTs is very important before any further use because as-prepared nanotubes typically contain traces of metal catalysts as a remnant of the synthesis procedure.

One of the most commonly used techniques for the purification of CNTs is the oxidation using strong acid treatments which permit removal of a large part of the metallic impurities and the possibility of breaking the tubes into smaller fragments that generate carboxylic functions at the tips and around the sidewalls [7]. Alternative methods of purification have been explored including chromatography, centrifugation, filtration or other chemical derivatization techniques [8–13]. These purification techniques increase also the nanotube solubility which make them easier to separate from the insoluble impurities. Dispersibility of CNTs in aqueous media is a fundamental prerequisite to study their biological applications. CNT are practically insoluble in any type of solvent and some strategies have been developed for linking the chemical moieties on the tubes to facilitate their applicability. Carboxylic functionalization can lead to further derivatized by reacting with amines to form the corresponding amides [14]. Another method for the preparation of functionalized CNTs is the 1,3-dipolar cycloaddition of azomethine [14]. All the above reactions work efficiently with both SWCNTs and MWCNTs. Functionalized CNTs may be sufficiently dispersible and soluble in aqueous and organic solvents. A number of different molecules, such as fullerenes, porphyrins, and metal nanoparticles have also been included in the internal space of CNT due to hydrophobic interactions [15,16]. CNT based composites with a metal or a metal complex could also be used to act as a potential delivery vehicle for the pharmaceuticals for diagnostic purposes.

Of special annotation for this work is the fact that CNTs have recently been considered as potential candidates for biomedical diagnostic and clinical purposes [17]. Therefore, along with their electronic properties, the biocompatibility of CNTs is a very promising new development for these applications.

Biological molecules can be combined with functionalized CNTs to form multifunctional hybrid materials with unique properties that will offer an intriguing combination of properties derived from both. Much progress has been made in developing functionalization schemes for carbon nanotubes to conjugate biological functions to this novel 1D material [18]. Intracellular delivery of biological molecules by carbon nanotube transporters should be significantly facilitated if efficient strategies can be devised to release biological cargos from nanotube walls via biologically triggered bond cleavage of nanotube bio-conjugates.

The present review will focus on the potential of CNT materials to deliver different types of therapeutic molecules such as proteins, drug compounds and nucleic acid into various cells, tissues and organs.

2. Discussion: application of CNTs within the scope of bioactive molecules

The functionalized CNTs with amines and carboxylate functional groups can also be modified or functionalized with therapeutic agents to create a CNT-based composite endowed with some kind of pharmacological activity. Nanotubes able to carry therapeutic moieties along with optical or other probes for imaging, and/or specific recognition signals for targeting, can offer options in the treatment of cancer and other types of complex diseases where activity is required only at specific sites in the body [19]. The application of CNT as a delivery vehicle has become possible due to their ability to penetrate into the cells. CNT can be loaded with active biomolecules by forming stable covalent bonds or supramolecular assemblies based on noncovalent interactions [19]. Once the vehicles are coming in contact with or internalized into various cells, tissues and organs they are able to express their biological function.

The important characteristics of an efficient delivery system are the ability to perform controlled and targeted delivery of the biomolecules. For this purpose, biomolecules should be released at an appropriate rate and location while maintaining their biological and chemical properties. Researchers have found that functionalized CNTs can cross the cell membrane [20,21] as due to their tiny size the cells do not recognize them as harmful trespasser.

Several technological problems must be addressed before proceeding with any kind of practical application, mainly of the pharmaceutical development nature in physiological conditions, the degree of aggregation of CNTs in vivo, the correct timing and location of drug release. However, considering the vast possibility of combinations offered, carbon based nanotubes are rich in technological platforms for the development of candidates for simultaneous diagnosis, transport and targeted delivery of biomolecules [22,23].

2.1. CNT as a delivery vehicle for protein and peptide

Protein delivery is a powerful technique for experiments in live cells for the studies of protein–protein interactions, protein interference with antibodies, intracellular trafficking and various biological functions. Most available agents or vehicles dedicated to the protein or peptide delivery allow efficient crossing of the plasma membrane but poor release ability into the cytoplasm. Numbers of recombinant proteins are available to offer the possibility to study protein function and develop therapeutic candidates with intracellular targets. For such a mechanism to be efficient, the delivered protein needs to cross the plasma membrane and to be efficiently released in the cytoplasm. Successful delivery therefore largely depends on the robust protein – (delivery vehicle) interaction which can efficiently transport the protein inside the cytoplasm. Since the functionalized CNTs appear to be non toxic for the cells, they can be considered as a new tool to deliver peptides, peptidomimetics or protein into the cells. Therefore, these systems can help to solve transport problems for pharmacologically relevant compounds that need to be internalized and may have potential therapeutic applications.

To determine whether CNTs could be used to deliver biologically active molecules into the cell, Pantarotto et al. [21] prepared the FITC–SWNT conjugate, **1**, and the FITC–peptide–SWNT composite systems, **2** (FITC, fluorescein isothiocyanate, a fluorescent molecule). The group showed the peptide responsible for the G protein (guanine nucleotide-binding proteins) function, covalently linked to SWNTs, penetrates into the cell. G proteins are an important family of proteins involved in signal transduction from many hormones and neurotransmitters. Peptide from α subunit (α_s) of the G_s protein corresponding to the amino acid sequence 384–394 was chosen for that experiment. The capacity of the fluorescent-labeled CNTs **1** and **2** to penetrate into the cells was studied by epifluorescence and confocal microscopy. Human 3T6 and murine 3T3 fibroblast cells were used for the testing and cultured at 37 °C. Both types of functional carbon nanotubes (**1** and **2)** were added to the cells at room temperature and incubated at 37 °C for 60 min. Fig. 1 shows the epifluorescence and confocal microscopy images of the 3T3 and 3T6 cells treated with different concentrations of CNTs **1** and **2**. From the image it is evident that conjugate **1** accumulates mainly in the cytoplasm. FITC alone and FITC-peptide, used as controls, are not able to enter into the cells under the same experimental conditions. The confocal analysis (Fig. 1B) clearly shows the presence of the fluorescent labeled compound inside the cell

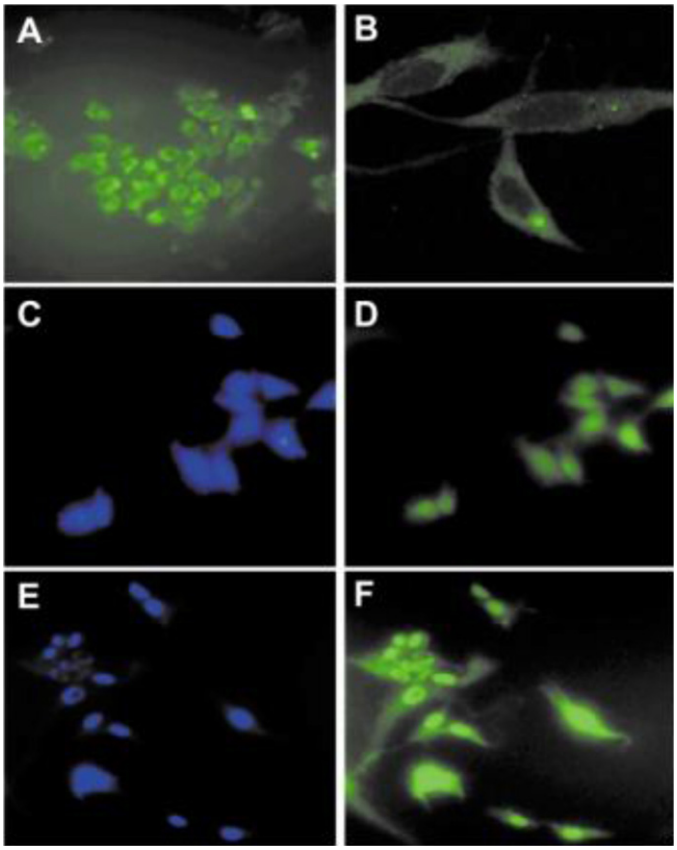

Fig. 1. Epifluorescence (A) and confocal microscopy (B) images of 3T3 cells incubated at 37 °C with CNT 1, respectively. Epifluorescence microscopy images (C, D, E and F) of 3T6 cells incubated at 37 °C with CNT 2.

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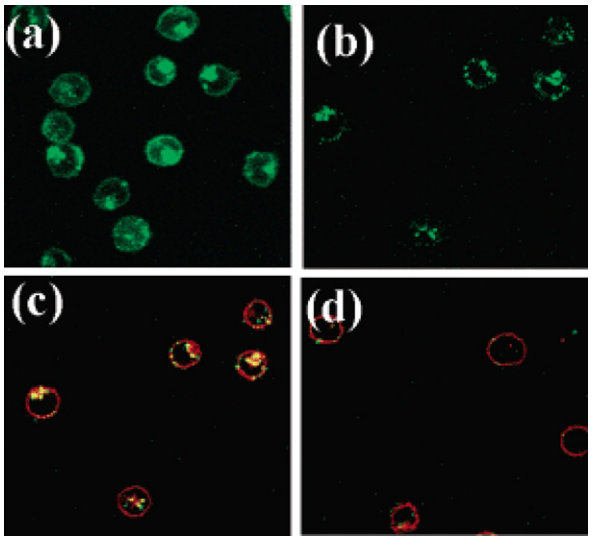

Fig. 2. Confocal images of cells after incubation in solutions of SWNT conjugates: (a) after incubation in **2**, (b) after incubation in a mixture of **4** (green due to SA) and the red endocytosis marker FM 4–64 at 37 °C (image shows fluorescence in the green region only), (c) same as b with additional red fluorescence shown due to FM 4–64 stained endosomes, (d) same as b after incubation at 4 °C. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

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and not stuck outside the membrane. Fig. 1D and F shows that peptide–carbon nanotube conjugate **2** is also able to cross the cell membranes and it distributes inside the nucleus (Fig. 1C and E). The fluorescence intensity at different times of incubation was also measured. After 10 min the fluorescence was as intense as after 60 min, suggesting that saturation is achieved very rapidly. According to the authors the translocation pathway of the functionalized CNTs across the cell membrane is inconclusive. The passive uptake mechanisms like endocytosis cannot be suggested for this process since the internalization is not affected by temperature or the presence of endocytosis inhibitors. CNTs behave like cell penetrating peptides (CPPs) [24–27]. The cellular translocation mechanism of CPPs is still unclear [28,29]. Generally, these compounds are able to reach the cell nucleus, due to their cationic character and the presence of amino acid sequences responsible for the nuclear localization signal. Water soluble CNTs (**1** and **2**) do not contain these motifs. However, while the destination of the CPPs is cytoplasm the peptide–SWNT derivative (**2**) goes mainly into the nucleus. The reason for this different selectivity is still unknown.

Kam et al. [30] reported their findings on the uptake of SWNT and SWNT-streptavidin (a protein with clinical applications in anticancer therapy) conjugates into human promyelocytic leukemia (HL60) cells and human T cells (Jurkat) via the endocytosis pathway. To visualize the interaction of nanotubes within cells, fluorescently labeled nanotubes were synthesized using 5-(5-aminopentyl)-thioureidyl fluorescein with SWNT (**1**). Fluorescently labeled nanotubes (**2**) were incubated with HL60 cells for 1 h at 37 °C. Confocal microscopy revealed fluorescence on the surface and in the cell interior (Fig. 2A). SWNT (**1**) was again functionalized with biotin-LC-PEO-amine (biotinyl-3,6,9-trioxaundecanediamine) to form SWNT (**3**), biotin-LC-PEO-amine, a hydrophilic, biocompatible tether has been demonstrated to covalently react with the previously generated carboxyl groups of the nanotubes, which was then incubated with fluoresceinated streptavidin (SA) to form SWNT-biotin-SA conjugate (**4**). HL60 cells were incubated with SWNT-biotin-SA conjugate (**4**) to evaluate the cellular uptake of the attached protein. Intense fluorescence had been observed inside the cells (Fig. 2B) which indicated the internalization of SWNT-biotin-SA conjugate (**4**). A systematic study as a function of time of cell incubation of 4 found uptakes increased with longer incubations. Upon increasing the concentration of 4 in the incubation solution, a monotonic increase in the cellular fluorescence was observed. To examine the potential toxicity of SWNT, HL60 cells were incubated with 1, 2, 3, and 4 and observed after 24 and 48 h. In the case of 1, 2, and 3, no appreciable cell death was observed. These results indicate that the functionalized SWNT themselves exhibit little toxicity to HL60 cells. The SWNT-biotin-SA conjugate (4), however, was found to cause extensive cell death with HL60. The results described above with HL60 cells appear to be general with other cells as well including Jurkat, Chinese hamster ovary and 3T3 fibroblast cells. The uptake pathway is consistent with endocytosis mechanism. The biocompatibility of SWNTs provides the basis for new classes of materials for protein delivery applications.

The interaction between nanostructured materials and living organisms is of fundamental and practical interest for the determination of biocompatibility and potential applications of nanomaterials in biological settings. The pursuit of new types of molecular transporters is an active area of research, due to the high permeability of cell membranes to foreign substances and the need for intercellular delivery of molecules via cell-penetrating transporters for protein, drug or gene therapeutics.

SWNTs are generic intracellular transporters for various types of proteins noncovalently and nonspecifically bound to nanotube sidewalls. In a detailed study Kam et al. [31] reported the intracellular protein transport and uptake through CNT carriers for various

mammalian cell lines, including HeLa, NIH-3T3 fibroblast, HL60 and Jurkat cells. The proteins investigated include streptavidin (SA), protein A (SpA) [Protein A is a surface protein originally found in the cell wall of the bacteria *Staphylococcus aureus*], bovine serum albumin (BSA), and cytochrome *c* (cyt*c*). Fluorescent (Alexa-fluor 488) labeled proteins were conjugated with —COOH functionalized SWNT and incubated with HL60, Jurkat, HeLa and NIH-3T3 cells in different incubation conditions at 37 °C. Intracellular internalization of protein-SWNT conjugates were investigated by confocal microscopy imaging and flow cytometry. The authors reported that the fluorescence was mainly originated within the cell interior. The fluorescence level was low for the fluorescent tagged protein alone (without CNT) compared to the fluorescent molecule-protein-SWNTs conjugated system suggesting that proteins were unable to traverse across cell membranes whereas, protein bounded SWNTs were effective in transporting proteins inside cells. The internalization of the noncovalently bound proteins via oxidized SWNT transporters follow the endocytosis mechanism [30]. Binding and intracellular protein transporting by SWNTs appeared similar for small- to medium-sized proteins whereas cellular uptake of protein-SWNT conjugates was poor and nearly nonexistent for a large protein such as human immunoglobulin. The study also confirmed that cell proliferation and cell viability were unaffected by the internalized carbon nanotubes.

To investigate the generality of carbon nanotubes for the transportation of proteins [Bovine serum albumin (BSA) and streptavidin (SA) labeled with an alexa fluor 488 dye] inside mammalian cells, Kam et al. [32] used two different mammalian cell lines, adherent HeLa cells and non-adherent HL60 cells. Control experiments were carried out through the incubation of cells in protein in the absence of nanotubes. In parallel experiments, HeLa and HL60 cells were incubated with various noncovalent protein-SWNT conjugates (Fig. 1A) and analyzed by both flow cell cytometry and confocal fluorescence microscopy. The study confirmed that the noncovalent conjugations between proteins and SWNTs are sufficiently strong for entry as cargo-carrier complexes into living cells. This was shown by the comparatively small intracellular uptake of fluorescently tagged protein molecules without nanotube transporters. According to the authors, the transportation mechanism is energy-dependent endocytosis.

The internalized nanotubes were found to be biocompatible and nontoxic at the cellular level. At the present time, several fundamental issues remain to be addressed for the use of carbon nanotubes as potential biological transporters. One such issue is the entry mechanism that regulates the cellular internalization of SWNTs and their cargos. Kam et al. [30,31] have suggested that SWNTs traverse the cellular membrane through endocytosis, whereas Pantarotto et al. [21] have suggested an energy independent non-endocytotic mechanism that involves insertion and diffusion of nanotubes through the lipid bilayer of the cell membrane.

Liu et al. [33] reported the bio-distribution of radio-labeled SWNTs in mice. In an experiment they used phospholipids (PL) bearing polyethylene-glycol (PEG, molecular weights 2000 and 5400) functionalized SWNTs (PL-PEG-SWNT) for in vivo applications. A chelating agent, 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid (DOTA), was attached to the terminal of the PEG chains and conjugated with positron emitting radionuclide (^{64}Cu) and a peptide, cyclic Arg-Gly-Asp-D-Tyr-Lys [*c*(RGDyK)], a potent integrin $\alpha_v\beta_3$ antagonist. The effect of SWNT-PEG2000 and SWNT-PEG5400 were studied on mice bearing integrin $\alpha_v\beta_3$-positive U87MG tumours. It was found that both SWNT conjugates exhibited prominent uptake in the liver but lower uptake for SWNT-PEG5400 in comparison with SWNT-PEG2000. Blood sampling revealed that SWNT-PEG5400 exhibited a much longer blood circulation time than SWNT–PEG2000. Bio-distribution in various organs after killing the mice at 24 h post-injection revealed prominent SWNT uptake in the liver and spleen and low uptake in the tumour, muscle, bone and skin. It has been observed that the efficient targeting of integrin $\alpha_v\beta_3$-positive U87MG tumours through RGD-functionalization of SWNT-PEG5400 (SWNT-PEG5400-RGD) and SWNT-PEG2000-RGD showed only a slight increase in tumour uptake compared to that without RGD. Raman spectroscopy was used to detect SWNTs in the various tissues of a mouse by taking the advantage of the intrinsic optical properties of SWNTs. No toxicity or negative health effects (such as weight loss and fatigue) were found with mice injected with SWNT–PEG over monitoring periods of up to several months. The work established the function of SWNTs for in vivo applications. The unique one-dimensional shape and flexible structure of SWNTs enables a poly-valency effect and enhances tumour binding affinity.

2.2. CNT as the delivery vehicle for drug molecules

Nanotechnology received remarkable attention with an unprecedented enthusiasm because of its future potential that can literally revolutionize each field in which it is being exploited. In drug delivery, nanotechnology is just beginning to make an impact where the concept and ability to manipulate molecules and supramolecular structures for producing devices with programmed functions. Systems being used currently for drug delivery include dendrimers, polymers, liposomes and nanomaterials. Among them nanomaterials are promising mainly due to their high surface area and other size related benefits. Again, among nanomaterials carbon nanotubes are considered as superior due to the effective structures that have high drug loading capacities and good cell penetration qualities [34].

Interaction between covalently functionalized CNT with drug molecules is a new research field and still under exploration [35]. The development of functionalized carbon nanotubes to target and be taken up by specific cell populations without any consequences for healthy tissues would be of fundamental importance in cancer treatment [18,36]. The molecular targeting of carbon nanotube delivery systems derived with a therapeutic agent becomes feasible if an active recognition moiety is simultaneously present at the surface of the nanocarrier [18]. In addition, attachment of a fluorescent molecule would provide optical signals for imaging and localization of the CNT-drug conjugates.

Methotrexate (MTX) is a drug widely used against cancer. A major disadvantage of MTX is its low cellular uptake [37,38]. Pastorin et al. [39] described a methodology for the introduction of two orthogonally protected amino groups on the sidewalls of CNT functionalized with fluorescein isothiocyanate (FITC) and MTX. MTX in conjugation with CNT represents a promising approach to overcome its limited cellular uptake by enhancing its internalization via the functionalized CNT [40]. The capacity of penetration of nanotube-drug composite to the cells (Human Jurkat T lymphocytes) at 37 °C was monitored by epifluorescence and confocal microscopy. The presence of the fluorescence probe on the tubes allowed the analysis of its internalization and intracellular distribution. Fig. 3(A–C) shows the epifluorescence and confocal microscopy images of the Jurkat cells after being treated with the composite. It is evident that the composite material accumulates into the cytoplasm (Fig. 3B). The confocal analysis (Fig. 3C) clearly indicates the presence of the fluorescence labeled compound inside the cell and also localized around the nuclear membrane. The dose and time dependence of the internalization process was also studied and Fig. 3D shows the flow cytometry analysis of the cells treated with three different amounts of composite for 24 h, which evidenced that the fluorescence signal is proportional to the dose. The rapid internalization of the MTX by carbon nanotubes could be advantageous for an improved efficiency of the drug action.

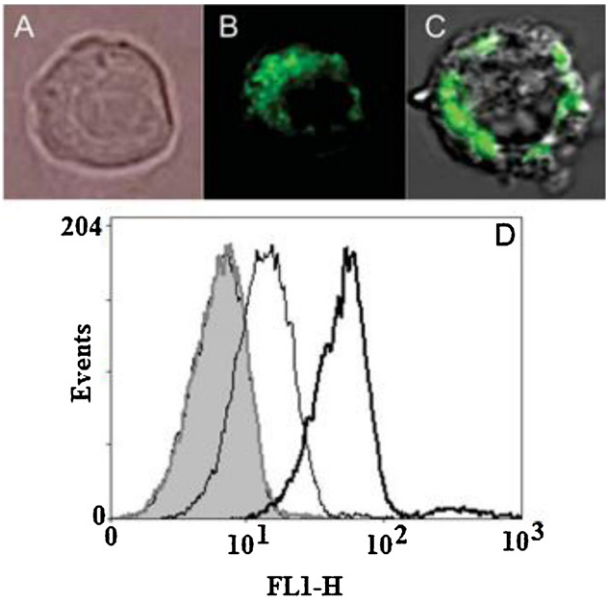

Fig. 3. Bright field (A), epifluorescence (B) and confocal (C) images of Jurkat cells incubated for 1 h at 37 °C with nanotube-drug composite. (D) Dose–response of the internalization after incubation of Jurkat cells for 24 h at 37 °C with increasing amount of nanotube-drug composite. FL1-H corresponds to FITC intensity.
Adapted with permission from Ref. [39]. Reproduced by permission of The Royal Society of Chemistry.

Theoretically, the use of functionalized CNTs for drug delivery would require the introduction of different functionalities on the external surface of the CNTs. Multiple functionalization of the CNTs has been reported recently [41]. Wu et al. [35] reported the strategy for the introduction of two different functional groups to CNTs. The methodology allowed the selection and control of the attachment of active molecules to the sidewalls and tips of the CNTs. This approach enabled for simultaneously linked fluorescent probes (Fluorescein) to the CNTs for tracking the uptake of material as well as an antibiotic moiety, Amphotericin B (AmB), as the active molecule, with the formation of SWNT composite. AmB is considered to be the most effective antibiotic in the treatment of chronic fungal infections [42,43].

The toxicity effects of MWNT composite and AmB alone (as a control) on human Jurkat lymphoma T cells indicated the conjugation of AmB to CNTs reduced the toxic effects of the antibiotic on mammalian cells. At the highest doses, more than 40% of the cells died in the presence of AmB alone, whereas all the cells remained alive upon treatment with MWNT composite. The longer incubation times did not increase the percentage of dead cells. All the Jurkat cells remained alive after treatment with MWNTs composite for 4 and 16 h. The uptake phenomenon of MWNTs composite was very fast as maximum fluorescence was observed after only 1 h of incubation. The researchers suggested the mechanism of penetration is not mediated by endocytosis. The functionalized CNTs behave like nanoneedles and pass through the cell membrane without causing cell death. This type of mechanism was recently confirmed by Cai et al. who showed that the nanopenetration of cell membranes seems to be a unique feature of CNTs [44].

Water-soluble SWNTs with poly-(ethylene glycol) (PEG) functionalization allow high degrees of π-stacking of aromatic drug molecules such as doxorubicin with ultrahigh loading capacity. Doxorubicin is an anthracycline antibiotic (Fig. 4) commonly used in the treatment of a wide range of cancers. Binding of molecules to nanotubes and their release can be controlled by varying the pH level of the medium.

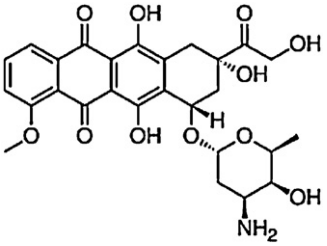

Fig. 4. Doxorubicin, [(7S,9S)-7-[(2R,4S,5S,6S)-4-amino-5-hydroxy-6-methyloxan-2-yl]oxy-6,9,11-trihydroxy-9-(2-hydroxyacetyl)-4-methoxy-8,10-dihydro-7H-tetracene-5,12-dione], an anthracycline antibiotic and works by intercalating DNA.

Liu et al. [45] introduced the concept of "partition functionalization" of SWNTs, i.e., depositing multiple chemical species, such as, poly-(ethylene glycol), chemotherapy drug (doxorubicin) and fluorescence molecule (fluorescein) on the wall of the nanotube. Binding of molecules to nanotubes and their release exhibit novel diameter dependence and can be controlled by varying the pH. SWNTs were functionalized by the surfactant [phospholipid (PL)-PEG, ~120 poly-(ethylene oxide) (PEO) units], PL-SWNT, or by PEGylation (~220 PEO units) of —COOH groups on oxidized SWNTs generated by nitric acid treatment. Doxorubicin (DOX) was attached to both kind of functionalized nanotube at pH 9. On the basis of optical absorbance data and molar extinction coefficients of DOX and SWNTs, it was estimated ~50 DOX molecules were bonded to each 10 nm length of SWNTs. The amount of doxorubicin bound onto SWNTs was pH-dependent and decreasing from a loading factor of ~4 to ~2, and to ~0.5 as pH was reduced from 9 to 7 and 5, respectively.

SWNTs (PL-SWNT) without DOX exhibited no toxic effect when incubated with U87MG cancer cells. DOX loaded SWNTs (PL-SWNT-DOX) induced significant U87MG cell death and cell apoptosis similar to free DOX although the IC_{50} (half-maximum inhibitory concentration) value for PL-SWNT-DOX was higher than that of free DOX. The half maximal inhibitory concentration (IC_{50}) is a measure of the effectiveness of a compound in inhibiting biological or biochemical function. IC_{50} is a quantitative measurement that indicates how much of a particular drug or other substance (inhibitor) is needed to inhibit a given biological process by half. DOX-loaded SWNTs were transported inside cells by nanotube transporters via endocytosis mechanism. According to the authors the potential advantages of using SWNTs as a drug carrier compared to free drug alone is the ability to target delivery for selective destruction of certain types of cells and reducing the toxicity to non-targeted cells.

A cyclic RGD peptide (a recognition moiety for integrin $\alpha_v\beta_3$ receptors) was again conjugated on the terminal groups of PEG on SWNTs to demonstrate the targeted delivery of doxorubicin. An enhanced doxorubicin delivery to integrin $\alpha_v\beta_3$ positive U87MG cells by RGD-conjugated SWNTs was also evidenced compared to those observed in cells treated by PL-SWNT-DOX without RGD. The PL-SWNT-RGD-DOX showed an enhanced cell-killing effect toward U87MG cells, with a lower IC_{50} value than that found prior to RGD conjugation, owing to specific RGD-integrin recognition and enhanced cellular uptake of the SWNT drug.

2.3. CNT as the delivery vehicle for nucleic acids

Gene therapy is being considered as a potential medical revolution for the treatment of hereditary diseases as well as for acquired diseases. In live cells gene delivery and expression is a complex biological phenomenon and that has been applied for gene identification and gene therapy. The optimum delivery of genes in diseased tissue is a challenge in both viral and non-viral gene therapy

applications. CNT are some of the most recent nanomaterial constructs to be developed as gene delivery vectors, stemming from both their attractive physicochemical features as well as the initial observations that they can be internalized in mammalian cells very efficiently.

To investigate the ability of carbon nanotubes for the transportation of DNA inside cells Kam et al. [32] used two different mammalian cells lines, adherent HeLa cells and non-adherent HL60 cells, and incubated with DNA-SWNT conjugates. SWNTs were first conjugated with the fluorescently labeled 15-mer-oligonucleotide [CATTCCGAGTGTCCA-*X*, where *X* = Cy3 (water-soluble fluorescent dyes of the cyanine dye family)]. The cellular uptake of the DNA-SWNT conjugates by HeLa cells was observed by confocal fluorescence microscopy after incubation at 37 °C (Fig. 5A). The conjugations between DNA and SWNTs are sufficiently strong for entry as cargo-carrier complexes into living cells. A systematic investigation of the cellular internalization mechanism and pathway for SWNT conjugates was also reported by the authors. Endocytosis, a general entry mechanism for various extra-cellular materials and being an energy dependent uptake [46–48], is hindered when incubations are carried out at low temperature (4 °C instead of 37 °C) or in ATP (adenosine triphosphate) depleted environments [49]. Cellular incubations with SWNT conjugates were also carried out at 4 °C and with cells pretreated with sodium azide (NaN_3) in parallel with regular incubation conditions. Treatment with NaN_3 is known to disturb the production of ATP in cells, thus hindering the endocytotic pathway. The fluorescence levels observed with confocal microscopy from cells (after incubation in SWNT conjugates at 4 °C (Fig. 5B) was negligible and ATP depletion by NaN_3 (Fig. 5C) was very low. This indicates that internalization mechanism for the uptake of SWNT conjugates into living cells is an energy-dependent endocytosis procedure.

Carbon nanotubes, a well-studied nanomaterial, have been investigated for their ability to interact with living systems. Gao et al. [50] reported on the conjugation between amino-functionalized multi-walled carbon nanotubes (NH_2-MWCNTs) with *p*EGFP-N1 plasmid DNA and their interaction into the human cells. DNA binding by NH_2-MWCNTs was due to an electrostatic interaction between the positively changed NH_2-MWCNT and negatively charged plasmid DNA. They functionalized MWCNTs with several other chemical groups (carboxyl-, hydroxyl- and alkyl groups) and found that only positively charged NH_2-MWCNTs could bind to anionic plasmid DNA and deliver it into mammalian cells quite efficiently.

Scanning-electron microscopy (SEM) image indicated that a sufficient amount of NH_2-MWCNTs bound on the cell surface which is due to the interaction between the positive charge of NH_2-MWCNTs and the negative charge of the cell membrane but only a small amount of nanotubes were found to be internalized into the cytoplasm.

The NH_2-MWCNTs were used as a carrier of *p*EGFP-N1 plasmid DNA for the delivery into human umbilical vein endothelial cells (HUVEC). Lipofectamine 2000 (a delivery agent for DNA or siRNA with excellent transfection performance for protein expression and gene silencing) was used as a positive control. The cultures were incubated for a further 48 h in order to allow for GFP-gene expression and the transfected cells were then imaged under a fluorescence microscope.

From the fluorescence microscopy image (Fig. 6) it is clear that NH_2-MWCNT, like Lipofectamine 2000 (Fig. 6B), efficiently delivered the exogenous GFP gene into cells (HUVEC) (Fig. 6A). Fluorescence was not observed with control cells that were incubated either with pEGFP-N1 (alone) or carboxyl-, hydroxyl- or alkyl modified MWCNTs (Fig. 6C). Although the transfection level of NH_2-MWCNTs was lower than that of Lipofectamine 2000, no cytotoxicity toward mammalian cells was observed. The above findings suggest that the positive charge of the amino group on NH_2-MWCNTs is a critical factor for gene delivery and these functionalized nanotubes could be used in biological research and gene therapy.

Exploration of the biological and medical applications of carbon nanotubes (CNTs) is a rapidly expanding field for the research of biology and applied medicinal science in conjunction with material science [20,21,30,51–55]. The use of CNTs as carriers of biologically active molecules holds great promise [22,56]. Functionalized carbon nanotubes are interesting materials for engineering a novel gene delivery system [**57**]. Pantarotto et al. reported [58] the ability of cell penetration by ammonium-functionalized CNTs (*f*-CNTs) in conjugation with plasmid DNA. In an experiment, (*f*-CNT)-plasmid DNA composite and DNA alone were incubated to the HeLa cells. The gene expression was found ten times higher for functionalized nanotube associated with DNA than those achieved with DNA alone (without nanotube). The TEM study revealed the incorporation of *f*-CNTs inside the cell (Fig. 7). Gradual magnifications of Fig. 7B and C indicate the cellular internalization of the *f*-MWNTs. The interaction between *f*-MWNT and the cell membrane (uptake mechanism) is clearly visible in Fig. 7D. According to Kueltzo and Middaugh [28] an endocytosis process could not be the mechanism of cellular uptake of CNTs due to its semi-rigid and elongated form. According to the authors [28] the entry mechanism of carbon nanotubes is a spontaneous process in which they behave like nanoneedles and pass through the cell membrane without causing cell death [59]. Molecular dynamics simulation data also suggested that hydrophobic nanotubes with hydrophilic functional groups can spontaneously insert into a lipid bilayer [60]. Such mechanistic modelling fits well with the experimental observations on the interaction between *f*-CNTs and plasma membranes (Fig. 7D).

From the above observations and experimental findings it can be concluded that compared with single-wall carbon nanotubes (SWCNTs), MWCNTs have many advantages in terms of easy

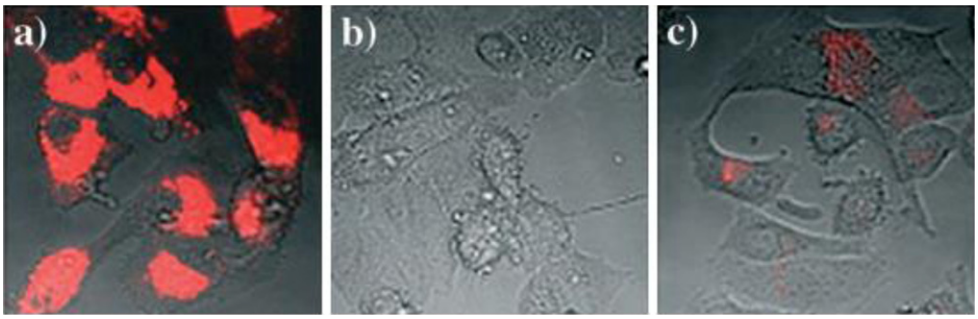

Fig. 5. Confocal microscopy images of HeLa cells after incubation in fluorescently labeled DNA–SWNT at (A) 37 °C, (B) 4 °C and (C) after pretreatment with NaN_3. Adapted with permission from Ref. [32]. Copyright 2004 John Wiley and Sons.

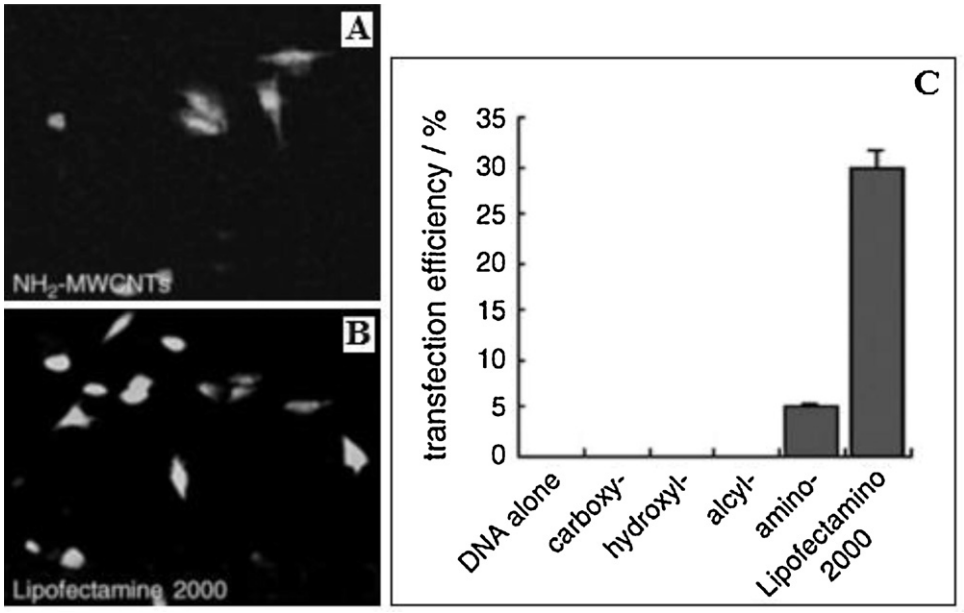

Fig. 6. Fluorescence microscopy image of the HUVEC cells that were transfected with *p*EGFP-N1 by using (A) NH_2-MWCNTs and (B) Lipofectamine 2000 (200× magnification). (C) Evaluation of the transfection efficiency of *p*EGFPN1 plasmid by using MWCNTs that were modified with different chemical groups.
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functionalization and biocompatibility. This suggests that MWCNTs can become a new promising reagent for the delivery of biomolecules.

The possibility of improving the transfection efficiency of nanotubes with shorter or smaller size, and higher charge density has also been discussed previously [19,20]. Further elucidation of the mechanism of cellular uptake of DNA–nanotube complexes will contribute to the optimization of carbon nanotubes in terms of their length, diameter, charge density, and solubility – as vehicles for gene delivery.

3. The fate and toxicity of carbon nanotubes under the physiological system

Very few reports have been published on the bio-distribution of chemically functionalized nanotubes into animals. According to Wang et al. [61] and Singh et al. [62], functionalized SWNTs behaved

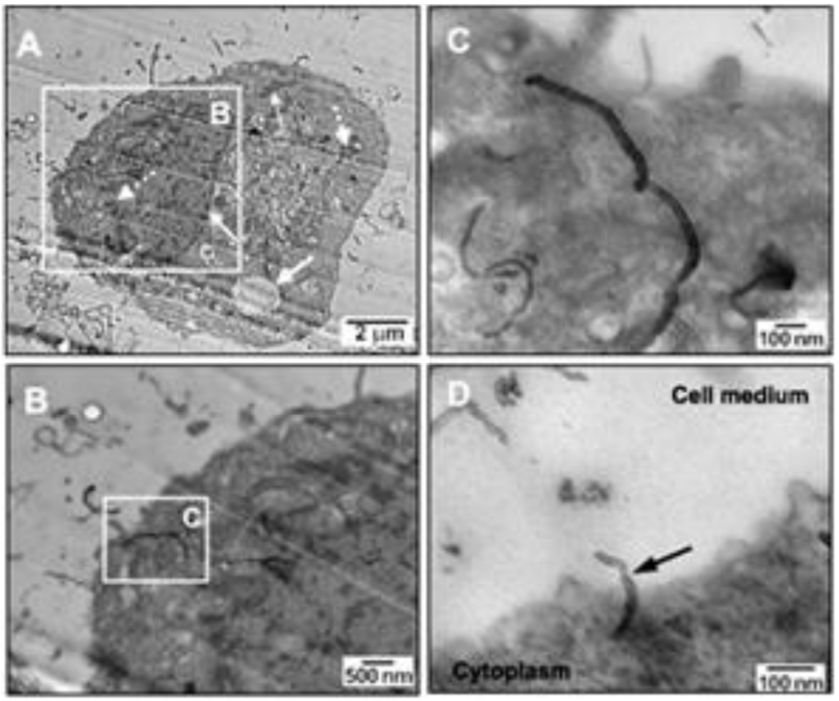

Fig. 7. TEM image of ultrathin transverse section of HeLa cells treated with *f*-MWNTs. (A) The entire cell, (B and C) two gradual magnifications, (D) a multi-walled carbon nanotube crossing the cell membrane.
Adapted with permission from Ref. [56]. Copyright 2004 John Wiley and Sons.

like small molecules in mice and freely cleared through the urine with little uptake by the liver or other organs of the reticuloendothelial system (RES).

Toxicity study is an essential part of the research when nanostructured materials are used for biomedical applications. Lacerda et al. [63] observed that the entry process of the intravenously injected multi-walled carbon nanotubes into the blood circulation system of the mice took place rapidly and within 6 h almost all of the nanotubes were eliminated through urine and very few were found in the lungs, liver and spleen. A micro single-photon emission tomography scanner was used to detect the nanotubes, which had been labeled with a radioisotope. In a separate study, Liu et al. [64] used Raman spectroscopy to track SWNTs wrapped with polyethylene glycol (PEG) in mice. In contrast to the study by Lacerda et al. [63], they found SWNTs in the intestines, faeces, kidneys and bladders of the mice and there was relatively slow excretion (about 2 months) via the renal and biliary pathways. This result is consistent with recent work on radio-labeled SWNTs by Yang et al. [65]. Schipper et al. [66] have made further progress by injecting noncovalently and covalently PEGylated SWNTs into the bloodstream of immune deficient mice and examining the acute and chronic toxicity over a period of 4 months. Throughout the study, no unusual behaviour was seen in either the mice injected with SWNTs or in the control mice. This was further confirmed by microscopic examination of the tissues isolated from the mice. Moreover, Raman mapping of the mouse tissues showed that both non-covalently and covalently PEGylated SWNTs persisted in the liver and spleen four months after injection, with a stronger signal seen in the liver but little or no inflammation.

The above studies provide some interesting observations and should be regarded as exploratory studies rather than the last word on the safety of carbon nanotubes. The medicinal application of carbon nanotubes is a fascinating topic still its infancy and the safe and auspicious administering of nanotube-related materials would need thorough investigations to determine issues such as safe dosage levels, side effects, and re-entrant disorder that are generally being applied as a measure of safe practice to novel medicines and treatments.

4. Conclusion

Delivery of biologically active molecules into the living cell or organism is a rapidly growing area for the treatment of various kinds of diseases. Commonly used systems for the delivery of bio-molecules are dendrimers, polymers and liposomes. Recently, carbon nanotubes have shown their efficiency as a delivery agent due to its high loading capacities and good cell penetration qualities. Specifically, carbon nanotubes have been used to deliver therapeutic molecules to targeted cells and organs in a safe manner which generates a low immunogenic response and is low in toxicity. Kam et al. [31,32] have suggested that SWNTs traverse the cellular membrane through endocytosis, whereas Pantarotto et al. [21] have argued in favor of an energy independent non-endocytotic mechanism that involves insertion and diffusion of nanotubes through the lipid bilayer of the cell membrane. Detailed work to establish the cellular uptake mechanism and pathway for SWNTs is currently lacking.

The scope of current data being produced on the many varied applications of nanostructured materials in biomedicine suggests that this research field holds promise to deliver far-reaching and unsurpassed breakthroughs in the diagnosis, treatment, control and curtailment of modern-era diseases and medical deficiencies.

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