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Carbon nanotubes part II: a remarkable carrier for drug and gene delivery

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Abstract

Introduction—Carbon nanotubes (CNT) have recently been studied as novel and versatile drug and gene delivery vehicles. When CNT are suitably functionalized, they can interact with various cell types and are taken up by endocytosis.

Areas covered—Anti-cancer drugs cisplatin and doxorubicin have been delivered by CNT, as well as methotrexate, taxol and gemcitabine. The delivery of the antifungal compound amphotericin B and the oral administration of erythropoietin have both been assisted using CNT. Frequently, targeting moieties such as folic acid, epidermal growth factor or various antibodies are attached to the CNT-drug nanovehicle. Different kinds of functionalization (e.g., polycations)

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have been used to allow CNT to act as gene delivery vectors. Plasmid DNA, small interfering RNA and micro-RNA have all been delivered by CNT vehicles. Significant concerns are raised about the nanotoxicology of the CNT and their potentially damaging effects on the environment.

Expert opinion—CNT-mediated drug delivery has been studied for over a decade, and both *in vitro* and *in vivo* studies have been reported. The future success of CNTs as vectors *in vivo* and in clinical application will depend on achievement of efficacious therapy with minimal adverse effects and avoidance of possible toxic and environmentally damaging effects.

Keywords

biomolecule delivery; carbon nanotubes; drug delivery systems; gene delivery; toxicology

1. Introduction

The new field of nanotechnology is expanding in terms of both the level of interest and the number of applications day by day. Nanomedicine is an area of nanotechnology with particular promise and includes the construction of nanoscale drug carriers for the targeted delivery of small molecules to a specific cell type or tissue [1]. Nanosized delivery vehicles may also improve the activity of a cargo biomolecule (i.e., antigens, proteins, enzymes and nucleic acids) by controlling release rates, as well as protecting the cargo from metabolism or degradation, or through targeted delivery that can reduce side effects [2].

In recent years, nanomaterials (NMs) have been well studied as drug carriers, therapeutic agents and diagnostic tools. There has been a huge technology push, leading to the development, successful testing and even clinical approval of several different kinds of nanocarriers. However, there is still a significant lag in their commercialization, especially due to the lack of international regulatory guidelines for evaluating the safety of NMs and growing public concern about their toxicity [3].

A drug delivery system (DDS) is usually designed to improve the pharmacological and therapeutic properties of conventional drugs and to overcome problems such as limited solubility, drug aggregation, poor biodistribution and lack of selectivity and to reduce normal tissue damage. Among the currently available delivery systems, which include liposomes, emulsions, polymeric micelles and micro-particles, carbon nanotubes (CNTs) have recently gained popularity as potential drug carriers, therapeutic agents and diagnostic tools [4,5].

An important advantage of using nanovehicles for drug delivery, especially for anti-cancer drugs, is that using a nanocarrier can frequently bypass or overcome the multi-drug resistance (MDR) pumps that pump out cytotoxic drugs from cancer cells, and whose over-expression is the main reason why so many cancers rapidly develop resistance to chemotherapy [6]. Moreover, covalent and non-covalent functionalization of CNT also enhances its solubility and prevents its aggregation in biological systems [7].

Using CNTs for delivery of proteins has been reported by many researchers [8,9]; they can improve the penetration of protein into cells and also increase protein uptake by the cells [10]. On the other hand, for the treatment of diseases, genetic materials can be used to

replace, regulate, add and delete the expression of a specific gene [11]. Because the design of viral vectors is relatively expensive and might be toxic to normal organs, a variety of nanovectors have been explored as possible gene therapy vehicles [12]. The unique properties of CNTs such as high length-to-diameter ratio, easy chemical functionalization and good biocompatibility make them a suitable candidate for this purpose [8,13]. Different types of nucleic acids such as micro-RNA (miRNA) [14], small-interfering (siRNA) [9] and plasmid DNA (pDNA) [15] can be bound to CNTs and transferred into mammalian cells.

As CNTs have become more widely used in industrial manufacturing, concerns have been raised about the possible impact of this material on the environment. However, it should be noted that the possible adverse effects of CNTs may also be due to contamination with residual metal catalysts, polyaromatic hydrocarbon impurities and even toxic metals extracted from laboratory and manufacturing equipments [16].

In part 1 of this pair of reviews in Expert Opinion in Drug Delivery, we had discussed methods of synthesis, purification and covalent and non-covalent functionalization of the CNT. In this second part, we try to review the mechanism of CNT uptake by cells and cover CNT applications in the delivery of small molecules and drugs as well as different genetic materials and protein delivery. At the end of this article, we also discuss toxicology and environmental concerns that have been raised concerning CNTs.

2. Uptake mechanisms of CNTs by cells

CNTs are one of the best candidates for drug delivery vehicles as they can be taken up by a wide range of different cell types, and their high surface area allows them to be an efficient carrier of cargo molecules. For this reason, the cellular internalization of CNTs has been widely investigated in recent years. There is no single mechanism for CNT cellular uptake but rather three different mechanisms that can each predominate depending on several factors. These three different processes are: i) internalization by endocytosis; ii) internalization by phagocytosis; and iii) direct translocation through the plasma membrane [17–21]. Endocytosis describes the engulfment of a macromolecule by the cell (e.g., proteins and antibodies) through the formation of a vesicle that is then generally routed to endosomes and lysosomes. Phagocytosis is similar in principle to endocytosis, with the difference that the particles taken up are considerably larger (e.g., bacteria with dimensions of less than 1 μm) and the cell type responsible is often a professional phagocytic cell. It has also been reported that CNTs can behave similarly to cell penetrating peptides, which allow translocation through the plasma membrane of mammalian cells due to the presence of polycationic regions. It has been stated that cationic functionalized CNTs are similar to cell penetrating peptides both in charge and morphology and may penetrate that plasma more likely than undergoing endocytosis Figure 1).

Factors that govern the uptake mechanisms can be summarized as: nanotube length, degree of aggregation and surface coating. Variation in the specific properties of the CNT preparation and the particular cell type responsible can determine exactly which mechanism operates [17,22]. Moreover, the nature of the functional groups that are covalently or non-covalently linked to the CNT surface and the nanotube dimensions play a role in the

mechanism of interaction with cells [2,19,23]. The CNTs possess the capacity to be taken up by many kinds of mammalian and prokaryotic cells by an energy-dependent or energy-independent mechanism [24]. Single-walled CNTs (SWCNTs) can bind to various types of proteins, and this binding can govern the uptake by endocytosis into endosomes, which subsequently are converted to lysosomes by epithelial and mesenchymal cells, and by phagocytosis into phagosomes by professional phagocytes [25,26]. Phagosomes fuse with lysosomes leading to digestion of the contents. For instance, fluoresceinated proteins attached to oxidized SWCNTs functionalized with biotin were internalized via the endocytosis pathway [23,27]. Biomacromolecules such as proteins, antibodies or DNA on the CNT surface (carboxylated or amidated CNT) can lead to the formation of small bundles that are internalized via energy-dependent endocytosis [2,26].

Shorter multi-walled CNTs (MWCNTs, i.e., 1 μm) have been reported to penetrate the cell membrane more efficiently than the longer CNTs, which can inhibit their uptake by self-arranging into a coiled or bundled shape. The role of nanotube dimensions influencing the internalization mechanism in cells was confirmed in a recent study where it was demonstrated that the cell uptake mechanism of SWCNTs was different from that of MWCNT bundles. SWCNTs more often enter cells by direct penetration, whereas MWCNTs enter via endocytosis [2].

Uptake mechanisms are also different depending on the cell type involved. Whether the cells are phagocytic or non-phagocytic determines the type of uptake behavior due to their particular biological function. It has been reported that functionalized CNTs were internalized by non-mammalian fungal and yeast cells, which indicates that the functionalized CNTs can be taken up by different cell types [23,24].

Some studies have examined the effects of the coating on the surface of CNTs. SWCNTs functionalized by collagen, proteins, polymers, PEGylated lipid or block copolymers, ssDNA and Pluronic F108 copolymer can be internalized into mammalian cells [23,28]. The translocation of ssDNA into the nucleus of HeLa cells via endocytosis was observed for all earlier mentioned CNT-based delivery systems, whether ssDNA is linked covalently to CNT or non-covalently [29]. In these cases, the cells recognized the coating instead of the CNT backbone. These macromolecular coatings modify the inherent properties of CNTs by ‘hiding’ their structure [30]. However, Liu *et al.* showed that CNT could be taken up by the cells where the functional groups were electrostatically neutral or negatively charged at physiological conditions as well as cationic. The effects of different functional groups at the surface of functionalized CNTs have been studied using methods such as FACS, confocal microscopy and treatments, which inhibit energy-dependent internalization mechanisms. Cai *et al*. made a theoretical analysis of penetration pathways. They showed that amphiphilic nanotubes can penetrate through artificial lipid bilayers via an endocytosis pathway [18].

Endocytosis inhibitors such as genistein, filipin III, amiloride, lowering the temperature (4°C) and use of NaN_3 can all decrease the cellular uptake of CNTs in some cells such as A549, but not in every cell type tested. These inhibitors can, therefore, be used to distinguish passive and endocytosis-dependent cell uptake pathway [17,19].

One efficient way to observe CNT uptake inside cells is by taking advantage of their intrinsic near infrared fluorescence as shown by Cherukuri *et al*., who found that significant amounts of nanotubes could penetrate into macrophage cells without toxic effects. The internalized CNT could be observed by emission at wavelengths beyond 1100 nm. There are some new techniques termed ‘nanotube spearing and nanopenetration’, which were suggested by Cai *et al*. to increase CNT uptake into cells [18].

3. Drug delivery

To maximize the efficacy of a drug, the choice of the delivery system is of prime importance [32]. Conventional drug administration often fails due to lack of selectivity, limited solubility, poor distribution between different cells, inability of drugs to cross cellular barriers and the presence of efflux pathways in MDR cancer [6]. DDS can be designed to minimize drug degradation, increase bioavailability, allow targeting to specific cells and reduce the total amount of drug needed, thus decreasing toxicity and harmful side-effects. CNTs possess a large aspect ratio (ratio between the length and the diameter, which for CNTs generally is about 1000:1) compared to classical DDSs such as liposomes or polymer-based carriers, and this parameter has been investigated since the first demonstration of the capacity of CNT to penetrate into cells [32,33]. Using large aspect ratio CNTs, different moieties can simultaneously bind to them. This feature of CNTs makes it possible to design multi-drug carriers and antibody conjugated DDSs, which will have improved detection capability. However, it should also be noted that large aspect ratio CNT may be more toxic than small aspect ratio CNT.

The well-established safety profile of other vesicle-based carriers (particularly liposomes) has discouraged many researchers from investigating CNTs in the treatment of diseases other than cancer [34]. These delivery systems for cancer generally consist of three parts: functionalized CNTs, tumor-targeting ligands and anti-cancer drugs [35].

3.1 Small molecule delivery

3.1.1 Cisplatin—Bhirde *et al.* [1] attached the anti-cancer drug cisplatin (CDDP) together with EGF to single-walled nanotubes (SWNTs) to specifically target squamous cell carcinoma, using a non-targeted control (SWNT–CDDP without EGF). They used quantum dots (Qdot) as a luminescent reported to follow uptake. Using video imaging they showed that SWNT–Qdot–EGF administered to live mice was selectively taken up in the tumor region in < 20 min. *In vitro* tumor cells treated with SWNT–CDDP–EGF were also killed selectively.

Guven *et al.* [36] described the preparation and *in vitro* testing of a new ultra-short SWCNT (US–CNT)-based DDS for cancer treatment. In this study, CDDP, a commonly used anti-cancer drug, was encapsulated within US-CNT. Dialysis studies performed in PBS at 37°C demonstrated that CDDP release from CDDP@US-CNT could be controlled (retarded) by wrapping the CDDP@US-CNT with Pluronic-F108 surfactant.

Dhar *et al.* [37] used a different method to attach CDDP to SWCNTs as an anti-cancer DDS. A platinum (pt) (IV) complex with the structure c,c,t-[Pt(NH3)2Cl2(O2CCH2CH2CO2H)

(O2CCH2CH2CONH-PEG-FA)] (Cpd1), containing a folic acid (FA) derivative at an axial position, was prepared and characterized. Cpd1 was attached to the surface of amine-functionalized SWCNT-PL-PEG-NH2 through multiple amide linkages to produce a 'longboat delivery system' for the platinum warhead. This construct was designed to allow cell uptake and the release of CDDP upon intracellular reduction of pt(IV) to pt(II). Once inside the cell, CDDP reductively released from the 'longboat oars', entered the nucleus and reacted with nuclear DNA, as determined by platinum atomic absorption spectroscopy of cell extracts.

3.1.2 Doxorubicin—Doxorubicin (DOX) is an anthracycline antibiotic that is used in treating various kinds of cancers. DOX has poor biodistribution, poor ability to cross cellular barriers and poor selectivity after intravenous (i.v.) administration. Despite the mentioned limitations, DOX can be improved by using CNTs as a drug transporter because of the ability of CNT to trap small molecules in their hollow space or on the surface and to transport them through cell membranes [4].

Heister *et al.* developed a DDS by combining DOX, a monoclonal antibody and fluorescein, all attached on the sidewalls of oxidized SWCNT [38]. The monoclonal antibody recognized the tumor marker, carcinoembryonic antigen, and allowed the binding of DOX to the antigen target sites on cancer cells. The delivery of DOX–SWCNT complexes to WiDr colon cancer cells resulted in penetration into cancer cells, followed by the release of DOX to the nucleus, whereas SWCNTs remained in the cytoplasm [6].

The non-covalent linkage of DOX to CNT was recently investigated by Liu *et al.* They used non-covalently functionalized and covalently functionalized SWNTs with PEG and PEGylation of carboxylic groups, respectively. The DOX–CNT complexes showed cytotoxic activity against cancer cells. They also made a drug carrier with tumor-targeting peptides that provided reduced toxicity towards non-target cells (Figure 2; Table 1) [7].

A targeted DDS for DOX based on SWCNTs was designed with drug release triggered by changes in pH. The CNTs were derivatized with carboxylate groups and coated with a polysaccharide material and could be loaded with DOX that was bound at physiological pH (pH 7.4) but was released at low pH characteristic of lysosomes and certain tumor environments. They also showed that by manipulating the surface of the modified CNT through modification of the polysaccharide coating, both the loading efficiency and release rate of the associated DOX could be controlled. FA, a targeting agent for many tumors, was additionally tethered to the SWCNTs to selectively deliver DOX into the lysosomes of folate-receptor positive HeLa cells with much higher efficiency than free DOX (Figure 2; Table 1) [39].

Liu *et al.* designed an efficient DDS for DOX by using non-covalently functionalized or covalently functionalized SWCNTs [40]. SWNTs that had been functionalized with PEG had good stacking of aromatic molecules including DOX and fluorescein with ultra high loading capacity. The strength of stacking of aromatic molecules was dependent on nanotube diameter, leading to a method for controlling the release rate. The cyclic arginine–glycine–aspartic acid peptide was conjugated to soluble SWCNT to act as a ligand for

integrin avb3 receptors and to enhance delivery to integrin avb3-positive U87MG cells. There was no noticeable improvement in the delivery of arginine–glycine–aspartic acid–SWCNT–DOX when integrin avb3-negative michigan cancer foundation-7 cells were used as a control [7]. The supramolecular stacking of DOX on SWCNT for *in vivo* lymphoma therapy was studied by the same group. They investigated biodistribution, toxicity and efficacy. SWCNT–DOX was more efficient and less toxic in comparison to equimolar amounts of DOX (Table 1) [40].

Ali-Boucetta *et al.* demonstrated that copolymer-coated MWCNT could form non-covalent supramolecular complexes with DOX. The formation of such complexes decreased the intensity of the DOX fluorescence emission and occurred presumably via p–p interactions with the MWCNT backbone. They also showed that the DOX–MWCNT complex exhibited enhanced cytotoxic activity compared to both DOX alone and DOX–Pluronic complexes [41].

3.1.3 Taxane—Taxanes represent another class of antineoplastic drugs that have been conjugated to CNT as a new delivery platform for cancer therapy. Paclitaxel (PTX) is a representative molecule of this class that is water insoluble and highly prone to aggregation. Liu reported that SWCNT–PTX conjugates had a higher anti-tumor effect than clinical taxol used alone in a murine 4T1 breast cancer model, owing to prolonged blood circulation times and the enhanced permeability and retention (EPR) effect leading to higher accumulation in the tumor [42]. Due to the very high surface area per unit weight, SWNTs provide a higher capacity for drug loading, compared to conventional liposomes and dendrimeric drug carriers. In addition, the intrinsic stability and structural flexibility of CNTs may prolong the circulation time as well as improve the bioavailability of drug molecules that have been conjugated to them [10].

3.1.4 Methotrexate—In a similar approach, Pastorin *et al.* [43] prepared a new anti-cancer agent from methotrexate (MTX) with a fluorescent probe attached on the CNT sidewalls. MTX is a drug widely used against cancer; however, due to its low cellular uptake [44,45], high concentrations of the drug are required. The results showed that MTX–CNT was rapidly internalized by cancer cells. However, MTX conjugated to the CNTs was only as active as MTX alone in a cell culture assay. The stable amide bond between the MXT and the CNT could be the reason for lack of enhanced efficacy, which results in the drug being released too slowly into the cytoplasm. One solution to this problem could be to introduce a cleavable linker or a more enzymatically sensitive bond (Figure 2).

3.1.5 Gemcitabine—Yang *et al.* [46] delivered a MWCNT–gemcitabine conjugate to lymphatic vessels, exploiting the EPR effect together with the use of an external magnetic field. MWCNTs were first functionalized with poly acrylic acid and then decorated with magnetite nanoparticles ($FeO.Fe_2O_3$) by a co-precipitation step with Fe^{2+} and Fe^{3+}. After subcutaneous injection in rats and waiting for a period of 3 h, the conjugate was able to reach popliteal lymph nodes with no accumulation in the main body organs such as the liver, spleen, kidneys, heart and lungs, simply by the EPR effect. The same CNTs were loaded with the antineoplastic drug gemcitabine by physical adsorption. The system was guided *in vivo* by applying a permanent magnet on the projection surface of one popliteal lymph node.

Very high accumulation of gemcitabine was detected in the lymph nodes after 24 h, compared to gemcitabine alone [46].

3.1.6 Other small molecules—Epirubicin (EPI) is a highly efficient anti-cancer drug related to DOX. However, it causes cardiac toxicity and severe bone marrow suppression damaging hematopoiesis. The use of CNTs as a drug carrier for EPI altered the biodistribution of EPI and enhanced its effective concentration in the tumor. CNTs formed a supramolecular structure with EPI through π–π stacking. Acid-treated MWCNTs (c-MWCNTs) had higher EPI loading efficiency than the untreated CNTs. The amount of EPI release from c-MWCNTs in the acidic medium was 1.5-fold larger than that in neutral medium (Figure 2) [47].

Chen *et al.* designed biotin-functionalized SWNT conjugates as a DDS for the anti-cancer drug taxol. They studied cancer-specific receptor-mediated endocytosis of the conjugate, followed by efficient drug release and binding of the drug to its target (cellular microtubules) by confocal fluorescence microscopy analysis. The conjugate was specific for cancer cells over-expressing biotin receptors on their surface and released taxol molecules inside the cancer cells [10].

Yinghuai *et al.* [48] studied the utility of CNTs as delivery agents for the element boron used in boron neutron capture therapy. Substituted carborane cages were attached to the walls of SWNTs via nitrene cycloaddition followed by a treatment with sodium hydroxide to yield water-soluble carborane-conjugated SWNTs. Boron tissue distribution studies showed that there was an enhanced boron uptake and retention of the carborane nanotubes in tumor tissue compared to blood, lung, liver or spleen.

Some reports have used CNT functionalized with antibiotics to deliver these molecules to the desired cells. In particular, Wu *et al.* [49] used CNTs in the administration of amphotericin B (AmB). AmB is a potent antifungal agent for the treatment of chronic fungal infections but is highly toxic for mammalian cells. It needs a drug delivery vehicle to increase the solubility in water and avoid formation of aggregates. CNTs were covalently linked to AmB and a fluorescent dye in order to study cell uptake and the toxicity towards human Jurkat lymphoma T-cells and a variety of fungal cells. The CNT–AmB system had lower toxicity towards mammalian cells, while its antifungal activity was higher than AmB alone.

Lymphatic metastasis occurs extensively in cancers and is problematic even after extended lymph node dissection. A lymphatic targeted DDS was developed using magnetic MWCNTs, which successfully delivered the anti-cancer drug gemcitabine to lymph nodes with high efficiency under the guidance of an external magnetic field. No systemic toxicity was observed.

Liu *et al.* showed that SWNT–PTX conjugates had a higher anti-tumor effect than clinical taxol used alone in a murine 4T1 breast cancer model, owing to prolonged blood circulation time and EPR in the tumor [42]. Due to the very high surface area per unit weight, SWNTs provide higher capacity for drug loading, compared to conventional liposomes and

dendrimeric drug carriers. In addition, the intrinsic stability and structural flexibility of CNTs may prolong the circulation time as well as improve the bioavailability of drug molecules conjugated to them [10].

3.2 Protein delivery

Dai and Wender functionalized oxidized CNT with biotin and formed a complex with a fluorescent streptavidin. Subsequently, the biotin moiety can be easily conjugated to the oxidized SWNT via an amide linkage. The biotin–streptavidin system is particularly convenient due to the highly specific interaction between biotin and streptavidin. CNT covered by the protein was found in the endosomes of chinese hamster ovary and 3T3 cells. They also studied uptake by human promyelocytic leukemia (HL60) cells and human T cells (Jurkat) via the endocytosis pathway [26]. In a similar approach, this group tried to determine whether SWNT could act as a transporter for generic proteins or not. They exposed acid-treated, oxidized SWNTs to solutions of various proteins. Because of the existence of carboxyl groups on the SWNT surface and positively charged domains on the proteins, a strong non-specific, non-covalent interaction was observed. Also the complex did not affect the viability and proliferation of cells during internalization procedure, which was confirmed to be endocytosis. This pathway was independent of the process used for functionalization and attachment of proteins [50]. Kam *et al.* [23] also investigated the ability of SWCNT to enhance intracellular internalization of streptavidin, protein A, bovine serum albumin (BSA) and cytochrome c. By mixing a 0.025 mg/ml concentrated oxidized suspension of SWCNT with proteins, complete attachment was achieved. They used HL60, Jurkat, HeLa and NIH-3T3 cells in order to show a positive effect on protein internalization across cell membranes and this effect was general for small to medium-sized protein (molecular weight < 80 KD). However, there was almost no cellular uptake for large proteins [23].

Erythropoietin is an important peptide drug to treat certain types of anemia and regulate red blood cell production. Venkatesan *et al.* used CNTs as a DDS to allow oral administration of erythropoietin. Oral administration of peptides has problems with enzymatic degradation and poor uptake from the gut, but CNTs were proposed to overcome these limitations.

A novel CNT-based delivery vector using poly(lactic-co-glycolic acid) (PLGA) was designed to internalize different proteins into cells. The carboxylated CNT preparation was functionalized with PLGA and then 5 μg of protein (either BSA, fluorescent BSA or caspase-3) was added to this system. X-ray Diffraction, Ultraviolet, Fourier Transform Infrared spectroscopy and Transmission Electron Microscopy investigations proved the functionalization and protein attachment to CNTs. Chen *et al.* showed that this new system had a high transfection rate, reduced toxicity and a highly controlled drug release profile and was also stable for weeks at −20°C [10].

Table 1 summarizes additional reports similar to those mentioned earlier.

4. Gene delivery

A great deal of research effort has been directed toward treating diseases by introducing genetic material (nucleic acids) to replace, regulate, repair, add or delete a specific genetic target that is responsible for the manifestation of a disease. The therapeutic effects accomplished by successful gene therapy have led to the next generation of disease-modifying medical interventions, through a wide range of therapeutically active nucleic acids, including pDNA, siRNA and miRNA, which have all been used to either over-express a protein or inhibit its expression. Gene delivery is not only the most important but also the most problematic aspect of gene therapy. Gene delivery systems can be viral vectors or non-viral vector systems [11]. Viral vectors (retrovirus, lentivirus, herpes virus simplex, RNA viruses and adenovirus) can achieve high transfection efficiencies. However, the application of viral vectors in the clinical setting is hindered by concerns that they can actually cause diseases, including cancer; the limited capacity of genetic payload; systemic instability; poor capability to target specific cell populations and the expense of production [51]. The alternative, non-viral delivery vectors exhibit advantages over viral vectors in terms of relative safety; they are chemically controllable with reduced toxicity [24] and the ability to deliver genes without any size limitation. However, non-viral gene delivery methods have not been very successful clinically compared to viral vectors due to serious limitations, including incapacity to transverse nuclear membrane, low transfection efficiencies and poor transgene expression [9].

A variety of nanovectors have been explored as possible gene therapy vehicles, including peptides, liposomes, polymers, dendrimers, micro- and nanoparticles, CNTs and many others [52,53]. However, the *in vivo* therapeutic delivery of nucleic acids is challenging for a number of reasons, including lack of stability against endogenous enzymes and inherently poor ability to cross cellular membranes.

One of the methods for gene transfection is using what is called a ‘gene gun’. Three different stages have been defined in particle delivery using a gene gun: acceleration, separation and deceleration. In this technique, DNA-loaded micro-particles are injected into the target tissue by applying sufficient pressure that might cause damage to cells and tissues [54], as a lesser, insufficient pressure may not allow the micro-particles to penetrate the tissues [55]. To solve this obstacle, micro-needles (MNs) have been used to reduce this tissue damage. The length of the MNs influences the particle penetration depth that is important for delivery of micro-particles in different tissue [56]. The penetration depth of micro-particles also depends on the particle momentum as well as the particle size, velocity and density. Model experiments showed that raising the operating pressure and size of the particle would increase the particle momentum and lead to increasing the penetration depth. On the other hand, the length of the MN is the major variable to maximize the penetration depth [57]. Moreover, the MNs insertion force can be calculated to optimize the MN device for enhancing the targeted particle delivery and predict minimum needed force for insertion of MNs [58].

CNTs have been investigated for the delivery of nucleic acids due to their unique properties [8,52,53]. Their high length-to-diameter ratio, their propensity to act as a template,

biocompatibility and easy chemical functionalization make them ideal candidate for molecular transporter systems. The length-to-diameter ratio, nanotube surface area and type of functional groups are important factors that determine how nucleic acids interact with CNTs [13].

Complete dispersion of pristine CNTs is difficult, so various types of surface functionalization are used to increase the solubility and also to improve biocompatibility and the ability to deliver nucleic acids [8]. In general, positively charged CNTs, covalently functionalized with amine terminal groups, are used to complex and deliver nucleic acids [59].

Functionalized CNTs can be covalently (aminated or initially carboxylated) or non-covalently coated through physical adsorption with molecules (polyamidoamine dendrimers or the basic polymer polyethylenimine [PEI]). Multiple studies have explored different methods of modifying the surface of CNT for the improved delivery of nucleic acids, including pDNA [60–63], siRNA [9,53,60–68] and miRNA [14], into mammalian cells.

Phospholipid-coated CNTs, functionalized with amine-terminated polyethylene glycol (PL PEG2000-NH2), ammonium-functionalized CNTs dendron-CNT [66] and carboxylated-CNT [65] have all been reported to be efficient in siRNA delivery with low cytotoxicity and were efficient for siRNA and DNA delivery in human cell lines or primary cells. Both SWCNTs and MWCNTs have been used as nucleic acid delivery vectors; however, the best results were achieved with MWCNTs.

The desirable properties for nucleic acid delivery systems are high loading of genetic cargos, stability outside the target tissues or cells, ability to protect DNA against enzymatic degradation, intracellular release of nucleic acids, controllable gene expression and low toxicity. However, the correlation between properties of carriers and the choice of the genetic cargo to be delivered into cells has not been well studied. There are a large number of different genetic cargos to be delivered, including pDNA, siRNA and miRNA.

4.1 Plasmid DNA

pDNA was the first type of nucleic acid to be successfully transfected by CNTs *in vitro*. Pantarotto *et al.* demonstrated that CNT-mediated delivery of pDNA led to expression of marker proteins such as β-galactosidase in chinese hamster ovary cells at approximately 10 times higher levels than naked pDNA alone. Furthermore, their group found that the overall charge ratio (+/−) of CNT-NH3+/pDNA complexes was an important factor determining the level of gene expression. Furthermore, various types of surface-modified CNTs with a range of different, surface-modifications have been used [59], cationic glycopolymers, PEI [63], PAMAM hybrids [60] and ethylenediamine [61]. Different studies have reported successful *in vitro* transfection of pDNA; however, there is very limited evidence to date of *in vivo* use of this technology. Delivery of pDNA via nanoparticle–CNT (NP–CNT) hybrids has recently been reported in an *in vivo* canine model of restenosis [69].

Similar to the majority of nanoparticle-based delivery systems, the hurdles that CNT-mediated delivery of pDNA needs to overcome are: i) endocytosis; ii) escape from the

endosomal vesicle; iii) release of the DNA from the CNT; iv) translocation into the nucleus; v) DNA transcription; and vi) protein translation (Figure 3) [70].

4.2 Small interfering RNA

RNA interference is a post-transcriptional gene silencing process induced by siRNAs and miRNAs, which inhibit gene expression, typically by causing the destruction of specific mRNA molecules. In the past few years, much effort has been invested in the discovery of effective ways to deliver siRNAs into cells and tissues. The major advantage of siRNA over small drug molecules as a therapeutic is that the sequences can be quickly designed for extremely specific inhibition of the target gene of interest, and the advantage over use of the actual protein is that the synthesis of siRNAs does not require use of a cellular expression system, complex protein purification, refolding schemes or complex protein purification, and is relatively uncomplicated [71].

Since the first clinical trial of a siRNA-based drug began in 2004, rapid advances have been made in the development of siRNA therapeutics, and other trials are currently under evaluation [72]. Although siRNAs possess many valuable characteristics, they may activate the innate immune system [73]. Furthermore, the potential for an ‘off-target’ effect is another challenge [74]. The term ‘off-targeting’ refers to the inhibition of a gene, whose expression should not be targeted, due to the fact that different genes may share partial homology with the siRNA [75]. A solution reported by Jackson *et al.* used 2-*O*-methyl ribosyl group substitution at the guide strand to reduce silencing of most off-target transcripts with complementarity to the siRNA guide [76]. Delivery of siRNA during the therapy is also an important challenge in siRNA therapy. Because of their large molecular weight (MW c 13 kDa) and polyanionic and hydrophilic nature, they are unable to enter cells by passive diffusion pathways.

Many studies have reported that CNTs are viable platforms for delivering biologically active siRNA into cells [9,53,64–68,70]. Much of the earlier research using CNT-mediated siRNA delivery focused on silencing genes that suppressed the proliferation and growth of cancer cells, but other therapeutic applications of CNTs have been reported to transfect difficult cell types [77] such as skeletal muscle cells [65] and T cells [78]. The cellular uptake of siRNA by CNT has been found to depend on functionalization and PEG chain length [78].

Bartholomeusz *et al.* [64] focused on pristine SWNTs delivering siRNA sequences. They aimed to induce cell death in a range of *in vitro* cancer cell lines showing biological activity and a high degree of specificity. Delivery of siRNA *in vitro* by surface-modified SWNTs was demonstrated in breast cancer cells [70]. Similar results were reported in various other cancer cell lines with knockdown of mRNA reaching around 40% and protein levels were reduced by 70% [63,67,68,79].

In a recent study, Huang *et al.* reported that PEI-functionalized CNT increased the positive charge on the surface of SWNTs and MWNTs, allowing electrostatic interaction with the negatively charged siRNA and serve as non-viral gene delivery reagents [80].

Therapeutic activity was reported in the first proof-of-concept study using amino-functionalized MWNT:siRNA constructs in an *in vivo* human lung cancer xenograft model. Intratumoral administration of the MWNT: siRNA complexes resulted in biologically active 'siTOX' a cytotoxicity-inducing siRNA sequence, leading to delayed tumor growth and increased survival of the tumor-bearing animals.

Varkouhi *et al.* showed that two cationic functionalized CNTs (CNT–PEI and CNT–pyridinium) could deliver siRNA [67]. Functionalized CNTs:siRNA complexes had 10 – 60% cytotoxicity in human lung cancer cell line H1299 and 10 – 30% silencing activity. However, CNT–PEI and CNT–pyridinium did not show any added benefit regarding siRNA silencing activity and cytotoxicity.

Al-Jamal *et al.* demonstrated that perilesional stereotactic administration of anti-caspase-3 siRNA delivered by CNT into the CNS reduced neurodegeneration and promoted functional preservation before and after focal ischemic damage of the rodent motor cortex using an endothelin-1 induced stroke model [66]. This is the first report that siRNA delivered into the CNS via CNTs was able to produce biological and functional (motor rehabilitation) effects in an induced stroke model. Consequently, CNTs could be envisioned as a delivery platform for siRNA that can be utilized for the treatment of a variety of neurological disorders in a localized region of the brain and afford both therapeutic and functional recovery (Figure 3).

4.3 Micro-RNA

The term miRNA refers to a small non-coding RNA molecule that functions in transcriptional and post-transcriptional regulation of gene expression [81]. miRNAs function via base-pairing with complementary sequences within mRNA molecules, usually resulting in gene silencing via translational repression or target degradation [82]. Currently, little is known about the ability of CNTs to deliver miRNA sequences in an intracellular manner. To date, there is only one report describing the use of 'unzipped' MWCNTs as miRNA delivery vectors [14].

The Dong group reported that PEI-grafted graphene nano-ribbon (PEI-g-GNR), formed by longitudinally unzipping MWCNT, can effectively transfer a gene probe into cells. The transfer process was monitored for the specific recognition of 'locked nucleic acid molecular beacons' (LNA-m-MB) by miRNA in the cytoplasm, which led to their hybridization and induction of a strong fluorescent signal due to the separation of the fluorescent dye from the quencher attached to the LNA-m-MB. The PEI-g-GNR nanocarrier showed little cytotoxicity and could protect the LNA-m-MB from nuclease digestion or SSB interaction. The proton-sponge effect of PEI led to relatively high transfection efficiency via endosomolysis, which allowed the sensitive detection of the recognized target miRNA.

There is still incomplete knowledge about the molecular mechanisms and specificity of regulation of gene expression by miRNAs, so the lack of published studies on CNT-mediated miRNA delivery for gene therapy applications is not surprising, although their clinical application is promising (Figure 3) [83].

5. Toxicology and environmental concerns

As discussed earlier, the use of CNTs could be a promising approach for treatment of cancer and other diseases and their efficacy has been confirmed by several studies. However, potential CNT toxicity represents a major challenge as well as a growing concern for human use [84,85]. CNTs can accumulate in tissues, organs such as lungs, heart, testes [86] and brain [87], generate oxidative stress and cause cell damage. The functionalization of CNTs, their purity, size, length, diameter, surface chemistry and dispersion methodology are all factors that can determine and alter the levels of toxicity [88].

5.1 Effect of length and diameter of CNTs

It is thought that rod-shaped, long fiber or needle-like (large aspect ratio) CNTs are potentially more toxic to cells than shorter nanotubes with larger diameters (small aspect ratio) due to their larger contact area with the cell membrane during the membrane wrapping process of endocytosis or phagocytosis. This causes a large distorting force on the cytoskeleton of the phagocyte, leading to impaired clearance by phagocytosis and can lead to macrophage spreading onto the surface rather than internalizing it [84,85]. All CNTs are likely to be phagocytosed by macrophages, but small aspect ratio CNTs are more easily digested. Although in humans, oral and i.v. administration, inhalation and transdermal delivery are the most common drug delivery methods, the majority of *in vivo* studies investigating the toxicity of CNTs are based on intratracheal instillation and inhalation. Studies using oral and i.v. route are scarce. Inhalation of CNTs was shown to result in more severe inflammation compared to oral, i.v. or transdermal administration, but the most toxic route of administration is still not known and requires further investigation [89].

5.2 Types of CNTs and systemic toxicity

The toxicity of both SWCNT and MWCNT has been suggested to be dose-dependent [90,91]. The nanometer-scale diameter and fiber-like shape of CNTs resemble asbestos fibers and this resemblance has created concerns about whether these fiber-shaped particles could cause mesothelioma, a type of cancer occurring in the lining of lung and chest cavity that is almost exclusively caused by asbestos exposure [92]. Epithelioid granuloma and interstitial inflammation were observed upon intratracheal administration of SWCNT, whereas administration of MWCNT through the same route resulted in mesothelial proliferative lesions that were induced by inflammatory events in the lung and pleural cavity and were likely mediated by macrophages [93,94]. Functional respiratory deficiencies and decreased bacterial clearance were also reported in mice treated with SWCNT [90]. In a pilot study, intraperitoneal (i.p.) injection of long MWCNTs produced inflammation, foreign body giant cells and granulomas, which were similar to the foreign body inflammatory response caused by long asbestos fibers [95]. The results of this study suggested that the fibrous shape seems to dominate over simple graphene chemistry in the effects on the mesothelium. It is important to emphasize that i.p. injection of long asbestos fibers into the peritoneal cavity of rats has been demonstrated to cause mesothelioma in the long term [96], and the CNT study did not investigate whether the mice may also go on to later develop mesotheliomas [95]. If there is a link between inhalation exposure to long CNTs and mesothelioma in rats, it is not known whether there would be sufficient exposure to such

particles in the workplace or the environment to reach a threshold dose in the mesothelium. A recent 13-week whole body inhalation study on rats using different concentrations of MWCNTs demonstrated granulomatous changes (at 0.2 mg/m^3 in males, 1 and 5 mg/m^3 in females), focal fibrosis of the alveolar wall (at 1 mg/m^3 or higher for both sexes) and increased inflammatory parameters (concentration dependently in both sexes from 0.2 mg/m^3). Inflammatory infiltration in the visceral pleural and subpleural areas was induced only at 5 mg/m^3, and it was concluded that 0.2 mg/m^3 was the threshold for respiratory tract toxicity [97].

Upon i.p. administration of MWCNT to imprinting control regions (expressing genes from only one allele out of two) of female mice, the liver changed to a rounded shape and fibrous adhesions on internal organs as well as deposits on the surface of the liver and diaphragm were observed. Increases in the number of leukocytes, monocytes and granulocytes in the peripheral blood in addition to over-expression of pro-inflammatory cytokines/chemokines have been reported [98].

Several *in vitro* studies have demonstrated possible neurotoxic effects of CNTs, including a decrease in cell viability, induction of oxidative stress and apoptosis; however, it is worth mentioning that the uptake, localization and cytotoxicity of nanoparticles were dependent on the cell type [99,100]. A recent *in vivo* study, which better mimicked the possible effects in humans, showed that doses of 6.25 and 12.5 mg/kg/day SWCNTs in mice could lead to cognitive deficits, reduced locomotor activity, increased oxidative stress, inflammation, apoptosis as well as histopathological alterations in mouse brains but 3.125 mg/kg/day had either minor or no adverse effects [87].

MWCNT can also be teratogenic (causing birth defects), but depending on the route of administration (e.g., i.p. vs intratracheal) the teratogenic dose may be different [101].

5.3 Effect of impurities present in CNTs

Absorption routes in the skin allow molecules to travel in the gaps between the stratum corneum cells (intercellular), through the cells (transcellular) or through the hair follicle or sweat ducts (trans-appendageal). These routes may differ between different types of CNTs, due to differences in size, shape, surface modifications and composition. Purity of the CNT has been suggested to be an important factor for toxicity when administered dermally. When non-purified SWCNTs (30% iron) were exposed to mice skin, oxidative stress, depletion of glutathione, an increase of dermal cell number, as well as skin thickening (due to accumulation of polymorphonuclear leukocytes and mast cells) were observed [102]. However, Monteiro-Riviere *et al.* concluded that the effects of MWCNTs on the cells were not caused by the catalytic metals. Various groups have studied toxicity of CNTs on the skin, but there is still limited information available regarding the mechanism of toxicity and skin absorption [103].

5.4 The effect of CNT aggregation

As mentioned earlier, CNTs with needle-like structures (large aspect ratio) can escape phagocytosis and thus are more disposed to flow through capillaries and adhere to blood

vessel walls [104]. This phenomenon can induce platelet aggregation and vascular thrombosis. Radomski *et al*. demonstrated that infusion of both SWCNT and MWCNT induced thrombosis in rat carotid arteries; however, they proposed several different underlying mechanisms for their results [105]. One explanation is that CNT may mimic molecular bridges involved in platelet–platelet interactions, thus stimulating platelet aggregation [105].

5.5 Effect of CNT functionalization

Functionalization can improve the biocompability of CNT and thus minimize the toxicity and potential for environmental damage. CNTs, which normally aggregate into clusters, can be separated and coated with biocompatible molecules [106]. Cells exposed to SWNTs, PEGylated by various PL-PEG amphiphiles, exhibited neither enhanced apoptosis/neurosis nor reduced proliferation when tested in various cell lines [7,107]. The metal impurities such as Co, Fe, Ni and Mo that are left after synthesis are one of the main factors that determine CNT toxicity. Purification, functionalization as well as ultra-sonication can all eliminate these metallic impurities and reduce the unwanted effects [85,108].

5.6 Effect of CNT surface chemistry

As the size of particles decreases, the surface area exponentially increases and becomes more reactive toward the surrounding biological components and the potential catalytic surface for chemical reactions increases. Therefore, further optimizing CNT surface chemistry and geometry is also needed to achieve improved biocompatibility.

5.7 Effect of CNT dispersion method

The toxicity of CNTs is also dependent on the methods used to disperse them. The effects of various dispersion methods were investigated by Zhang *et al* [24]. They used dimethyl sulfoxide and a 1% anionic surfactant Pluronic F127 to study the cytotoxicity of 6-aminohexanoic acid derivatized SWCNTs (AHA-SWCNTs). The AHA-SWCNT aggregates were dispersed by surfactant treatment in the culture medium, which resulted in less cytotoxicity. Pluronic F127 (1%) is a nonionic surfactant capable of shielding AHA-SWCNT cytotoxicity by coating its surface and forming micelles in the medium that altered the AHA-SWCNTs surface properties. It may be capable of decreasing the AHA-SWCNT adsorption to the cell membrane by shielding some of the cell membrane receptors. Dimethyl sulfoxide does not effectively disperse the aggregates, and therefore, it does not affect the biocompatibility of AHA-SWCNTs.

6. Conclusion

CNT in both the single and multi-walled formats can be taken up by cells depending on the precise dimensions and the functionality of the sidewalls by three possible mechanisms. These are endocytosis, phagocytosis and membrane translocation. Professional phagocytic cells such as macrophages, dendritic cells, Kupffer cells, microglia, retinal pigmented epithelial cells and so on are likely to engulf CNT by phagocytosis, whereas epithelial and mesenchymal cells are more likely to take up CNT by endocytosis or transmembrane transport. Many studies using CNT as drug delivery vehicles have had the possible

improvement of cancer chemotherapy as a goal. Anti-cancer drugs such as CDDP, DOX and taxol can benefit from solubilization, disaggregation and targeted delivery. Cancer-specific ligands, such as monoclonal antibodies, growth factor receptor ligands or FA, can be attached to the CNT–drug construct to improve accumulation in the tumor and specificity. The systemic toxicity of these chemotherapeutics can also be reduced by CNT delivery. The other main cargo to be carried by CNT delivery vehicles is based on nucleic acids. pDNA, siRNA and miRNA have all been delivered by taking advantage of the unique properties of CNT. Major concerns have been raised about possible nanotoxicology of CNT. The resemblance between CNT and asbestos fibers has raised the possibility that CNT could cause mesothelioma. Overall, it is fair to say that, although the jury is still out on the long-term future application of CNT in drug and gene delivery, ongoing research will continue apace.

7. Expert opinion

The field of nanomedicine is expanding at an accelerating rate both in academic research laboratories and also in biotechnology companies. As perhaps the single most important discovery that has been made in the whole field of nanotechnology so far, CNTs are at the forefront of this research effort. The intense interest in this new allotrope of carbon, with such an interesting and recognizable molecular shape, has engendered a large number of studies with the goal of finding biomedical applications. There has been an enormous effort made to develop CNT into a bewildering variety of nanodevices using nanoelectronics, including biosensors and implantable power generators, and for tissue engineering applications. The electrical, optical, mechanical and thermal properties of CNTs make them a very attractive material for the detection and therapy of cancers and other diseases. The subject of this review is their use as drug and gene delivery vehicles, which is one of the newest biomedical applications of CNT. In a previous review, we covered the synthesis, purification, functionalization and derivatization of CNT to enable them to serve this purpose. However, much more research is still needed to explore and carefully define the limitations and opportunities of CNTs as drug and gene delivery systems *in vivo*. CNTs have been shown to be able to complex and transport different biomolecules both *in vitro* and *in vivo*. They have been proposed to easily transverse cellular membrane and translocate directly into cytoplasm of target cells due to their nanoneedle structure, utilizing both energy-dependent endocytosis or phagocytosis and energy-independent membrane translocation mechanisms without inducing cell death. The need for definition of the biological effects of the different structural and surface characteristics of preparations of CNTs is thought to be the most important factor for successful delivery of biomolecules. A lot more work needs to be done on the routes of administration of CNT in animals and in other well-established *in vivo* models such as flies, worms, caterpillars and zebrafish. The nanotoxicology aspects of CNT need to be thoroughly investigated to the satisfaction of all parties concerned, including environmentalists and ecologists. The concept of studying the pharmacodynamics and pharmacokinetics of CNT has not even been seriously raised as yet.

In the years to come, CNT will have to be measured up against a host of other nanotechnology-based DDS. The pros and cons of different systems will need to be systematically compared, and the strengths and weaknesses of each system will almost

certainly be different depending on the precise characteristics of the drug molecule or DNA sequence in question, not to mention the type of disease (cancer, infection, etc.) and the delivery route (i.v., interstitial injection, topical application). This will be a huge amount of work but will need to be carried out before CNT can play any clinical role in drug delivery.

In the burgeoning field of nanotoxicology, CNTs are also major players, possibly only rivaled in interest and popularity, medical and regulatory concern by Qdot that contain toxic elements like cadmium and selenium. However, scientific opinion appears to be sharply divided on the potential of CNT to cause acute and long-term toxicity. The similarity of CNT to the shape and dimensions of asbestos fibers has definitely raised concern about possible lung damage after inhalation in the short and long term. In the years to come, it is likely that validated conclusions will be arrived at on whether CNTs have a real future in clinical application of nanomedicine or not.

The most pertinent conclusion that can be obtained from multiple studies in 10 years of CNT-mediated transport and delivery of biomolecules is that biological efficacy has mainly been demonstrated *in vitro*. The future success of CNTs as vectors *in vivo* will depend on the achievement of efficacious therapy with minimal adverse reactivity and avoidance of possible toxic effects due to the characteristics of these particular nanostructures.

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Article highlights

- Carbon nanotubes (CNTs) have unique electrical, mechanical and optical properties and a high surface area that makes them suitable for attaching biological cargoes.
- CNTs are taken up into cells by endocytosis, phagocytosis or membrane translocation, depending on dimensions, surface functionalization and cell type.
- CNTs have been studied as drug delivery vehicles for anti-cancer drugs that can be targeted by attachment of tumor-specific ligands.
- Many *in vitro* studies and a few proof-of-principle *in vivo* studies have shown efficacy and lack of gross toxicity.
- CNTs can act as non-viral delivery vehicles for nucleic acid-based therapeutics, such as plasmids, small-interfering RNA and micro-RNA.
- Concerns have been raised about possible adverse effects on the environment and whether CNTs have acute and long-term toxicity.

This box summarizes key points contained in the article.

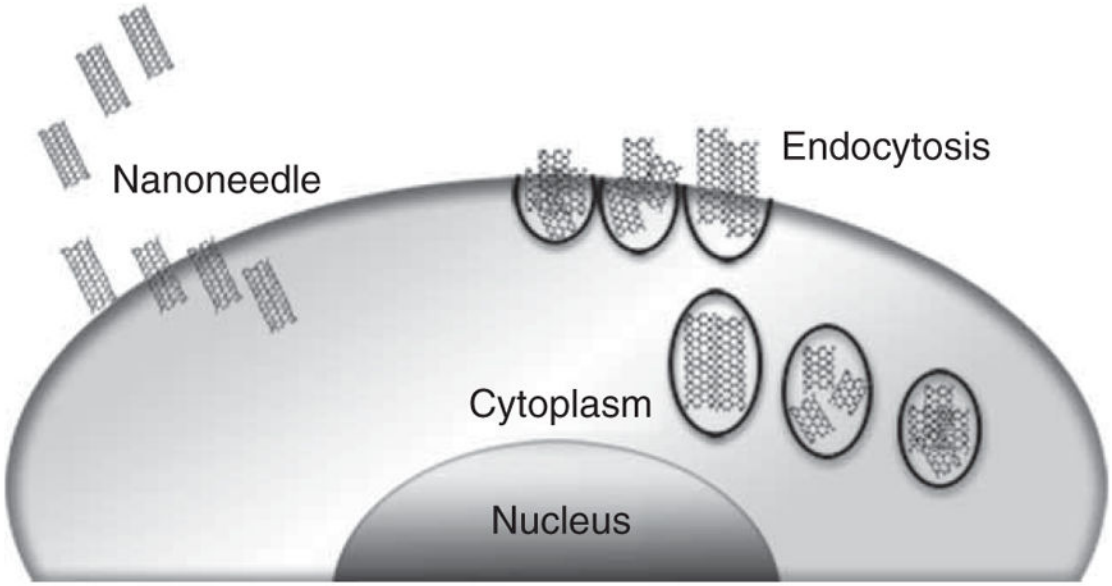

Figure 1.
Feasible internalization pathway for carbon nanotubes.

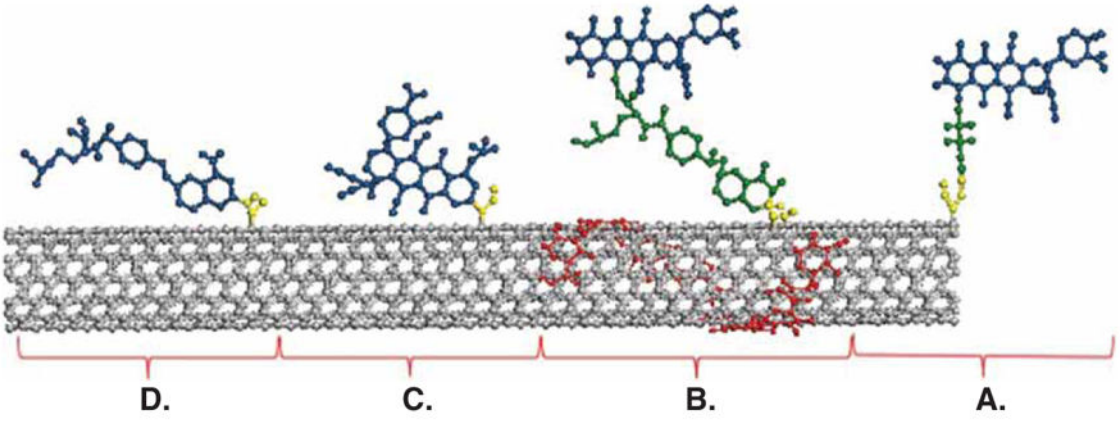

Figure 2. Drug delivery systems

(A) Covalent attachment of DOX to CNT by PEGylation of carboxylic acids; Liu *et al.* **(B)** Attachment of DOX to CNTs coated with polysaccharide; Zhang *et al.* **(C)** π–π stacking of acid-treated CNTs with epirubicin; Chen *et al.* **(D)** Methotrexate attachment to amino functionalized CNTs; Pastorin *et al.*

CNT: Carbon nanotube; DOX: Doxorubicin.

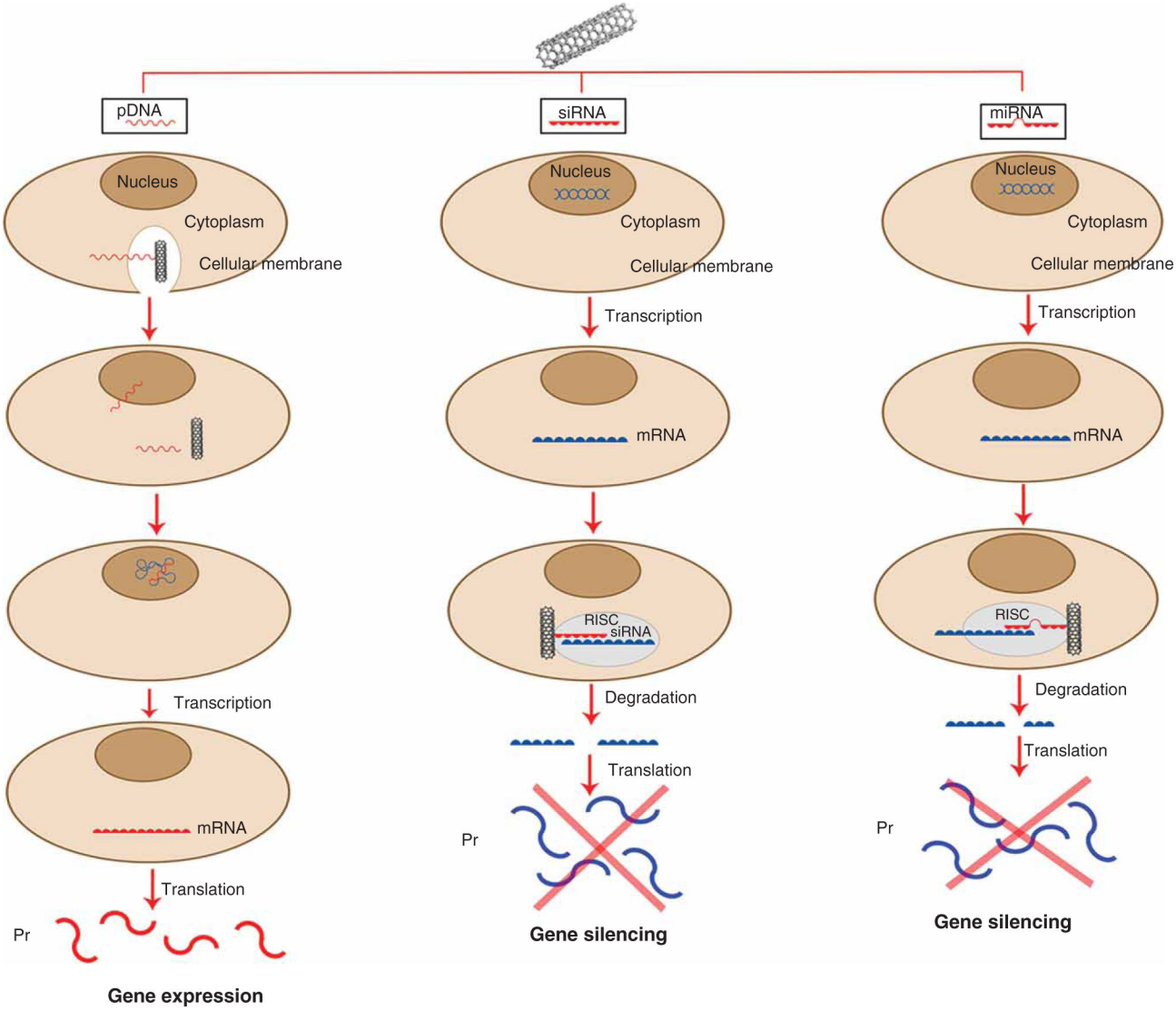

Figure 3. Gene expression and gene silencing mechanisms of pDNA, siRNA and miRNA attached to carbon nanotubes

miRNA: Micro-RNA; pDNA: Plasmid DNA; RISC: RNA–induced silencing complex; siRNA: Small interfering RNA.

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Table 1

Examples of DDSs.

CNT properties					Biological tests		
CNT	**Functional group**	**Drug**	**Release control**	**Targeting mechanism**	***In vitro***	***In vivo***	**Ref.**
SWCNT	Derivatizing CNT with carboxyl groups and coating with a polysaccharide material	DOX			–	Uptake and cytotoxicity in HeLa	[39]
SWCNT	Functionalization with PEG via π–π stacking	DOX	Acidic PH	–	–	Uptake and cytotoxicity in SCID Histological and treatment of lymphoma xenograft model	[40]
MWCNT	Covalently functionalized with amine terminated PAMAM dendrimers modified with FITC and FA	DOX	Acidic pH	FA against FR	Uptake and cytotoxicity in high and low FR expressing KB	–	[12]
MWCNT	Amino functionalized CNT with DEX mesylate	DOX	Acidic pH	DEX mesylate for nuclear targeting	Uptake and cytotoxicity in A549	–	[109]
SWCNT	Non-covalently functionalization with FA terminated methoxy-PEG	DOX	Acidic pH	FA against FR	Uptake and cytotoxicity in HeLa and 3 T3	–	[110]
MWCNT	Covalently functionalization with 1.2-dipalmitoyl-glycero-3-phosphocholine (DPPC) multimellar liposome	MTX	Cellular reductive environment	–	–	Uptake and cytotoxicity in RPMI 1640	[44]
MWCNT	Linked with EDBE conjugated with FA, HA or β-estradiol-17-hemisuccinate	MTX	Neutral PH	FA against FR HA against HR ES against ER	Uptake and cytotoxicity in A549, HeLa and MCF-7	–	[111]
MWCNT	Covalently conjugated with FA	GEM	Acidic pH	FA against FR	Cytotoxicity in MCF-7	Biodistribution and PK in albino rats	[112]
MWCNT	Mannosylation	AMB	–	Mannose to target macrophages	Uptake in J774	Biodistribution and toxicity in albino rats	[15]
MW/SWCNT	Form supramolecular complex with EPL via π–π stacking	EPI	Acidic PH	Changes the distribution of EPI and enhances its effective concentration at the tumor site	-	Uptake of therapeutic molecules	[47]

CNT: Carbon nanotube; DDSs: Drug delivery systems; DOX: Doxorubicin; EPI: Epirubicin; FA: Folic acid; MTX: Methotrexate; MWCNT: Multi-walled carbon nanotube; SWCNT: Single-walled carbon nanotube.