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Review Article

CARBON NANOTUBES AS CARRIERS FOR DELIVERY OF BIOACTIVE AND THERAPEUTIC AGENTS: AN OVERVIEW

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ABSTRACT

Nanostructures of carbon were first observed in 1952 which gained worldwide interest due to their various physicochemical properties. Carbon nanotubes are well-ordered, hollow nanostructures which are classified as Single-walled carbon nanotubes (SWNTs) and Multiple walled carbon nanotubes (MWNTs). Preparations of CNTs employ the following methods: electric-arc discharge (EAD), catalytic chemical vapour deposition (CVD), and laser ablation (LA). These methods produce pristine CNTs. The purification techniques used are chromatography, microwave assisted purification, high temperature annealing, microfiltration and oxidative purification. A major drawback of pristine CNTs is their insolubility in many solvents, especially water due to which they are incompatible with the biological systems. To integrate CNTs into biological systems, CNTs need to be functionalized. Functionalized CNTs have been shown to be able to cross cell membranes in many studies. The CNTs enter the cell by two possible mechanisms, that is, via the endocytosis pathway and via the endocytosis-independent pathway. CNTs have found wide applications in delivery of therapeutic agents such as peptides, proteins, siRNA, Nucleic acids, genes, vaccines and also in bone and neural tissue regeneration. The toxicity of CNTs is a major issue to address. Pristine CNTs are toxic due to their insolubility in water and metallic impurities. Functionalised CNTs have found to be biocompatible. This review attempts to highlight all the aspects of carbon nanotubes such as their structure, properties, synthesis and purification, functionalization, cellular uptake mechanism, applications and toxicity.

Keywords: Carbon nanotubes, f-CNT, Electric-arc discharge, Catalytic chemical vapour deposition, Laser ablation, Pristine CNTs.

INTRODUCTION

Many new drug delivery systems have been developed with aim to improve the therapeutic efficacy of many classes of drugs. The salient features of an efficient drug delivery system include its ability to perform controlled and targeted drug delivery.

Nanostructures of carbon (carbon nanotubes) were first observed in 1952. Nearly four decades later, carbon nanotubes (CNTs) were prepared formally and gained worldwide interest due to their mechanical, electrical, thermal, optical, and structural properties.[1] Since the discovery of CNTs, several reports on their actual and potential use in nanotechnology have been published.

Carbon nanotubes have been considered for several applications, ranging from ultra strong fibers[2] to field emission displays.[3] CNTs have generated great interest in biology.[4] CNTs can be employed in several biological applications, among which drug delivery appears to be particularly promising.[5, 6] Single-walled carbon nanotubes (SWNTs) have been extensively studied for their use as carriers for drugs and gene delivery *in vivo*.[7, 8]

Structure and properties

Structure

Carbon nanotubes are well-ordered, hollow nanostructures consisting of carbon atoms bonded to each other via sp2 bonds (C-C distance of 1.4 Å) which are stronger than sp and sp3 bonds rendering CNT's excellent mechanical strength and high electrical and thermal conductivity.

CNTs belong to the fullerene family of carbon allotropes particularly those which have high aspect ratio. These are hollow cylinders consisting of a hexagonal arrangement of sp2-hybridized carbon atoms and formed by rolling single or multiple layers of graphene sheets into seamless cylinders.[9] These cylindrical structures have two forms: single-walled carbon nanotubes (SWNTs) and multiwalled carbon nanotubes (MWNTs). SWNTs are composed of a single cylindrical graphene layer capped at both ends in a hemispherical arrangement of carbon networks. The inclusion of pentagonal and heptagonal C-C structures during the growth process enables the closure of cylinder. MWNTs comprise several to tens of concentric cylinders of graphitic shells, each one forming a SWNT. MWNTs generally have a larger outer diameter (2.5–100 nm) than SWNTs (0.6–2.4 nm) and consist of a varying number of concentric SWNT layers. The interlayer separation between the SWNT layers of MWNT is about 0.34 nm. SWNTs have a better defined diameter, whereas MWNTs are more likely to have structural defects. Owing to its structure, MWNTS are less stable nanostructure.[10] Both possess a high tensile strength, are ultra-light weight, and have excellent chemical and thermal stability.[11] CNTs have the ability to buckle and collapse reversibly due to high stiffness and resilience. The high C-C bond stiffness of the hexagonal network produces an axial Young's modulus of approximately 1 TPa and a tensile strength of 150 GPa, making CNTs one of the stiffest materials known, yet with the capacity to deform elastically under compression.[12]

Properties

The solubility of CNTs in aqueous solvents is a prerequisite for biocompatibility, hence CNT derivatives in therapeutic delivery should meet this basic requirement. Moreover CNT dispersions should be uniform and stable to obtain accurate concentration data. The solubilisation of pristine CNTs in aqueous solvents is a hurdle in designing CNTs as pharmaceutical excipients. The hydrophobic character of the graphene sidewalls, coupled with the strong p-p interactions between the individual tubes causes CNTs to assemble as bundles, reducing their solubility. To overcome the problem of aggregation of CNTs the dispersing medium should be capable of both wetting the hydrophobic tube surfaces and modifying the tube surfaces to decrease tube aggregation.

Four basic approaches have been used to obtain dispersion:

(1) Surfactant-assisted dispersion,

(2) Solvent dispersion,

(3) Functionalization of CNT sidewalls, and

(4) Biomolecular dispersion.

Surfactants in general can be useful for dispersing CNTs, although the chemical structure of the surfactant is important. Ham et al[13] determined that if adequate dispersibility is to be achieved, an alkyl chain length greater than 10 was required and dispersibility increased with increasing alkyl chain length. Moore et al[14] determined that within a series of non-ionic Pluronic surfactants (P103, P104, P105, F108, F98, F68, F127, F87, F77, F85) solubility, a measure by ultraviolet (UV) spectroscopy of individually dispersed

CNTs, generally increased with increasing molecular mass. The higher molecular weight surfactants and polymers increase the solubility of CNTs through steric stabilization by adsorbing surfactant/polymer onto the walls of the CNTs, thereby impeding aggregation. In case of ionic surfactant the charge of the head group, rather than the hydrophobic alkyl chain length is responsible for dispersion of CNTs. As the charge (zeta potential) increases, the dispersion is stabilized by the increased electrostatic repulsion. The current limitations of surfactant-based solubilisation are: the relatively low levels of solubility and that the surfactant often remains as an impurity in downstream processes.[15] Nevertheless, this approach could be potentially useful in pharmaceutical applications, where surfactants are routinely incorporated in formulations for improved delivery.

For solvent dispersion method, the organic solvents used to achieve dispersion are N,N′-dimethylformamide, N-methylpyrrolidone, chloroform, and dimethyl sulfoxide (DMSO).

However, these suspensions are stable only over a time scale of days.[13, 16]

In case of functionalization approach, dispersion is achieved by introducing polar functional groups on their surfaces which eliminate the Van der Waals forces between individual or bundles of CNTs. The most common functionalization approach is carboxylation by itself and the subsequent functionalization of the carboxylated intermediate into other soluble biocompatible products.[17] Some of the relevant functionalization techniques used include the 1,3-dipolar cycloaddition reaction and functionalization with polyethylene glycol moieties i.e. PEGylation.

DNA also have been used to disperse CNTs. Initial reports suggested that ssDNA-assisted dispersion of CNTs in solution depended on a nucleotide sequence rich in guanine and thymine. However, subsequent results indicated that this dispersion is not necessarily a function of nucleotide sequence but primarily a result of p-stacking of the ssDNA on the CNT sidewalls.[18] Covalent sidewall functionalization is expected to produce the most stable dispersion, because the dispersion becomes a function of the bound functional groups and the density of the bound groups.

Surfactant and biomolecular dispersion include physical wrapping of molecular units around the CNTs, which involves forces that are relatively weaker than those involved in covalent functionalization. The chemistry of solvent dispersion, however, is not clear.

Synthesis and purification

Synthesis

Method of synthesis of CNTs decides the physical properties, quality, quantity and type of CNTs produced. Thus choice of synthetic method depends on the intended use of the CNTs.

The major synthetic approaches include electric-arc discharge (EAD), catalytic chemical vapour deposition (CVD), and laser ablation (LA).

Electric-arc discharge (EAD): In the EAD setup, a plasmon is generated across carbon electrodes which results in the deposition of CNTs on a substrate. In its original configuration, the anode was constructed from pure graphite, resulting in MWNTs as the main product. However, the incorporation of nanometre-sized metal catalysts in the anode material yielded SWNTs. The limitations of EAD are its inability to produce either SWNTs or MWNTs exclusively, variability of CNT diameter, the tangled/bundled nature of the products, and the presence of metal impurities. This necessitates difficult and exhaustive purification stages by chemical and thermal treatment.[19]

Laser ablation: Laser ablation uses a laser beam to vaporize a graphite target mixed with a transition metal catalyst. As with EAD, LA can yield both SWNTs and MWNTs. Optimization of the vaporization of the target can be done by introducing another laser source which also minimizes the amount of carbon deposited as soot by the breakdown particles ablated by the primary laser.[20] The limitations of this technique are the narrow diameters of SWNTs produced and the presence of tangled ropes and bundles along with graphitic particles, metals, and carbonaceous impurities.

Catalytic chemical vapour deposition (CVD): Unlike LA and EAD, CVD synthesis involves a vaporized hydrocarbon typically mixed with an inert gas. This is fed into a furnace where it decomposes, depositing CNTs onto a substrate. The substrate is prepared by embedding nanometre-sized catalyst particles (e.g. nickel or cobalt) onto its surface. Various adaptations, such as plasma-enhanced CVD, thermal CVD, laser-assisted CVD, and high pressure CVD, have been made to the initial method. CVD and the CoMoCAT method (developed by Southwest Nanotechnologies Inc.) are two currently available methods that allow for direct synthesis and deposition of CNTs on patterned substrates and control of nanotube diameter. [21]

The length and diameter of CNTs are a function of the conditions of synthesis. Whereas the length of both SWNTs and MWNTs (typically tens of microns) are controlled by the length of the synthesis time (the longer the time the longer the length), the diameter of SWNTs is controlled by the size of the catalyst, which is typically 0.7 to 3.0 nm wide. For larger sized catalyst particles, SWNTs fail to grow and instead the larger diameter MWNTs are formed.[22]

Purification

Pristine CNTs are completely insoluble in many solvents, especially water which render them incompatible with biological systems. Therefore purification of pristine CNTs is must. Several methods have been used to purify the as-produced CNTs to the desired quality. Among them are chromatography, microwave-assisted purification, high-temperature annealing, microfiltration, and oxidative purification.[23-26] The purification of CNTs by oxidative treatment is the most widely used of the purification processes mentioned. It involves selective oxidation of impurities by refluxing in mineral acids to remove metal catalysts, and gas phase oxidation to remove carbonaceous impurities. These liquid-phase oxidative treatments routinely use HNO_3, HCl, $KMnO_4$, H_2SO_4, OsO_4, $K_2Cr_2O_7$, and several combinations of these and/or refluxing in water or H_2O_2.

Analytical techniques for evaluation

For pharmaceutical applications, a consistent protocol must be developed to evaluate the purity, dispersion, and physical and functional properties of CNTs. These properties include nanotube size and type, surface defects, electronic characteristics, mechanical strength, and thermal conductivity. Although various techniques have been used to characterize CNTs, there is no industry standard to evaluate the quality of produced CNTs.

Several techniques have been used to characterize the structure and morphology of CNTs, to determine the purity of CNT materials, and to establish the presence or absence of exogenously bound moieties onto the walls of CNTs. The most extensively used techniques are thermogravimetric analysis, scanning electron microscopy (SEM), transmission electron microscopy (TEM), atomic force microscopy (AFM), Raman spectroscopy, infrared (IR) spectroscopy, and nuclear magnetic resonance (NMR). Whereas TEM, SEM, and AFM have been used predominantly to qualitatively establish the general morphology of CNTs, IR spectroscopy, Raman spectroscopy, and NMR have been used to confirm the presence of functional groups on CNTs. [27]

Thermogravimetric analysis is used to quantitatively determine the amount of carbon and non carbon materials in bulk CNT samples, as well as CNT homogeneity and thermal stability. This is a nonselective method for assessing CNT quality, because the technique does not differentiate CNTs from metallic impurities present in the sample. It is therefore used in conjunction with other techniques.

TEM uniquely provides qualitative information on the size, shape, and structure of carbonaceous materials, as well as non-CNT structured impurities in a sample. TEM has also been used to image cellular uptake of CNT-drug composites and to determine the fate of the CNT component after cellular uptake.

SEM is used in the preliminary evaluation of CNT morphology. It is the most widely used technique to evaluate bulk productions of

CNTs. SEM is probably the only technique that can provide information on both CNT morphology and the metallic impurity content.

H^1-NMR has been used to monitor the synthesis and attachment of functional groups to CNTs.

IR spectroscopy is primarily a qualitative tool used to identify functional groups and the nature of their attachment to CNT sidewalls. It is a complementary technique to NMR, to confirm the presence of bonds between CNTs and attached moieties.[28]

No single analytical technique is capable of defining the purity of a CNT sample. Typically, CNT samples from commercial sources are accompanied by Raman spectra, elemental analysis data, and SEM/TEM micrographs.

Functionalization of carbon nanotubes

Pristine, as-produced, CNTs tend to bundle up or get tangled and are insoluble in most types of solvents making it difficult to use them in biological systems. Moreover, some CNTs without any functionalization have been shown to be cytotoxic. The cytotoxicity of pristine CNTs is due to the residual metal catalysts (impurity) and also the insolubility of pristine CNTs. Therefore, to integrate CNTs into biological systems, CNTs need to be functionalized. Functionalization can make CNTs soluble and improve their biocompatibility properties. In addition, through functionalization, bioactive agents can be conjugated to CNTs which can serve as a carrier for delivery of therapeutic agents.

Different functionalization strategies have been pursued and they can be divided into two main approaches:

1) Additional reactions to the sidewalls and tips of CNTs and

2) Oxidation followed by carboxyl based couplings.

In the first approach, additional reactions are employed to attach some organic groups to the sidewalls and/or tips of the CNTs. Briefly, purified SWNTs and aniline are mixed together. Under the pressure from the mixing force, the outermost nanotubes from the bundle is librated and is exposed to the reactive agent. Thus, the individual nanotube is covalently functionalized thereby preventing itself from bundling up with others. Preventing bundling up of CNTs is very important as more CNT surface area is available for the attachment with active molecules and the solubility of the CNTs can also be improved. The advantages of this functionalization strategy include its simplicity, its ability to produce highly soluble materials and its ease to implement in the industrial scale. However, this simple functionalization method has the disadvantage of not allowing for many desirable further modifications of the tubes.[29]

The second functionalization strategy is through oxidation and carboxyl-based couplings. In this method, the tube cap openings are created and holes in the side walls are formed by an oxidation process in which strong acids are used. In addition to opening tube caps and creating side wall holes, the oxidation introduced to the caps and side walls with carboxylic groups enhances the solubility of CNTs in aqueous solutions. The carboxylic groups also allow for covalent couplings with other molecules through amide and ester bonds. Through this method, CNTs can be conjugated with various functional groups such as bioactive agents like peptides, proteins, nucleic acids and therapeutic agents such as anti-cancer drugs. Importantly, by bonding with suitable groups, CNTs can become soluble in aqueous or organic solvents. The presence of carboxylic groups on the sidewalls of CNTs reduces Van der Waals interactions between the tubes, enabling separation of nanotube bundles into individual, separated tubes.[30]

Cellular uptake of CNTs

Functionalized CNTs have been able to cross cell membranes. The ability of CNTs to cross cell membranes has generated probe of CNTs in drug delivery strategies. In targeting the delivery of drugs to cells, drugs are first attached to the carrier by either covalent or noncovalent bonding. The drug-carrier conjugates are then directed to the targeted cells via passive targeting methods or active targeting methods. After reaching the targeted site there are two possibilities: the drug enters the cells without internalization of the carrier or both the drug and the carrier enters the cells. The latter internalization method has greater delivery efficacy as after entering cells, the intracellular environment degrades the drug-carrier conjugate releasing drug molecules inside the cells. On the other hand, in the former internalization method, the extracellular environment helps degrade drug-carrier conjugates and the drug then crosses the lipid membrane to enter the cells. CNTs with the ability to cross cell membranes are good candidates to serve as drug delivery carriers to cells with high efficacy.[31] It has been shown that there are two possible mechanisms of CNT internalization: (1) via the endocytosis pathway and (2) via the endocytosis-independent pathway.

(1) Internalization of CNTs via the endocytosis pathway

Endocytosis is a process in which cells engulf the material with a small area in the cell membrane to form a vesicle. The vesicle inside the cell then fuses with lysosomes forming endolysosomes. Then the lysosomal enzymes help digestion of the material. Since endocytosis is energy dependent, a low energy environment inhibits the endocytosis process. Studies have shown that CNTs can enter cells via the endocytosis process. Internalization of CNTs or CNT conjugates is usually monitored via fluorescence labelling. It was found that SWNTs conjugated with proteins (such as bovine serum albumin or biotin–streptavidin) or DNA (such as CATTCCGAGTGTCCA Cy3) were able to enter cells (human promyelocytic leukemia (HL60) cells or cervical cancer (HeLa) cells after incubation for 1 h at 37 °C but the protein or DNA alone was not found inside the cells under the same experimental condition. Especially, when incubated at 4 °C (which is the blockage endocytosis temperatures), or with cells pre-incubated with NaN3 (which is known to inhibit the production of ATP in cells), cellular uptake of either SWNTs or SWNT conjugates was significantly reduced. Endocytosis internalization of SWNTs was also directly visualized by using a "red marker" (FM 4–64) to stain endosomes formed around SWNTs during the internalization process. Furthermore, endocytosis internalization of CNTs was shown to be mediated by the clathrin receptor. Clathrin mediated endocytosis is the most common endocytosis route in which clathrin coated pits form under the plasma cell membrane and each pit then forms a closed vesicle inside the cell. SWNTs incubated with cells which were pre-incubated with either sucrose or a K^+-depleted medium before incubation greatly decreased cellular uptake.[32]

(2) Internalization of CNTs via the endocytosis-independent pathways

Experiments have shown that CNTs can cross cell membranes via the endocytosis-independent pathways. In one experiment, functionalized CNTs were labelled with a green fluorescent agent (FITC) and tracked by epiflourescence and confocal microscopy. After 1 h of incubating either flourescein alone (control) or the fluorescent labeled CNTs with cells (human 3T6 and murine 3T3 fibroblasts), the CNTs were found within the cells while the flourescein alone was not able to enter the cells. The internalization of these CNTs was not hindered by temperature (between 4° C and 37° C) or the presence of an endocytosis inhibitor (such as sodium azide). Therefore the CNTs might enter the cells via an endocytosis-independent pathway. Researchers also found CNTs in the cell membrane during the internalization process and the CNTs were found perpendicular to cell membrane. Therefore, CNTs might enter the cells via insertion and diffusion pathway.

The mechanisms of how CNTs can enter cells via insertion and diffusion are poorly understood. Some theoretical studies suggest a two step process in which, the tubes first are accommodated onto the lipid cell membrane and then oriented to adopt the transmembrane configuration. In this model, the internalization of nanotubes into the cells was spontaneous and was mediated by the lipid membrane and that the hydrophilic interactions and/or static charge interactions between the tubes and the lipid membrane drove the translocation of the nanotubes. CNTs can also enter cells under the application of external magnetic fields. Catalyst nickel particle residuals on the tips of CNTs produced by the plasma-

enhanced chemical vapour deposition method (PECVD) gives CNTs an ability to respond to an external magnetic field. Therefore, using an external magnetic field, it is possible to drive the CNTs to enter the cells bringing with it loaded cargos such as drugs or genes. However, not all CNTs produced by PECVD are able to respond to a magnetic field. It was observed that only CNTs which are less than 2 μm have Ni particles embedded on the tips of CNTs can be driven by a magnetic field into cells. CNTs longer than 15μm and that have Ni particles with aspect ratios of about 0.7 do not respond to a magnetic field. This method of inserting CNTs into cells was demonstrated on Bal17 B-lymphoma, ex vivo B cells, and primary neurons and showed a very high efficiency of delivering plasmid DNA into cells. The mechanism of this penetration method is to simply use a rotating magnetic field to drive the CNTs into the cells and then use a static magnetic field to force the CNTs deeper into the cells. This simple mechanism has resulted in a 107-fold increase in the delivery efficiency of CNTs into the cells.[33]

There are several factors that are crucial in cellular uptake of CNTs. First, surface properties of CNTs can greatly influence their interaction with cells and therefore their internalization into the cells. For example, there are hydrophobic and hydrophilic regions on cell membranes and the hydrophobic and/or hydrophilic interaction of the cells with CNTs will be influenced by the hydrophilicity of the tubes. Second, size and shapes of CNTs can also be important in their abilities to go into cells. CNTs which are well dispersed and have shorter lengths are more likely to be internalized by the cells than bundled CNTs or CNTs that have longer lengths.

Applications of CNTs

The search for new and effective drug delivery systems is a fundamental issue of continuous interest. A drug delivery system is generally designed to improve the pharmacological and therapeutic profile of a drug molecule. f-CNT has potential of being used as for the delivery of small drug molecules as it penetrates into cells.

Solubility and circulation time of drug can be increased by attaching drug to suitable carrier, thus the bioavailability of the drug can be maximized. The therapeutic efficacy of the drugs thus can be improved because of the facilitated transportation of the drugs to the desired tissues/organs.

Among the carriers for drug delivery SWNT are promising due to several factors like safety as compared to quantum dots, which have heavy metal compositions. SWNTs with diameter less than 30 nm can be completely eliminated from mice. CNTs posses capability to penetrate cell membranes. This provides a route for delivery of cargoes into the cytoplasm as well as the nucleus of cells.[34]

The intrinsic spectroscopic properties of nanotubes, including Raman and photoluminescence, provide additional advantages for tracking, detecting, and imaging to understand drug delivery efficacy *in vivo*.

The general process of drug delivery using a carbon nanotube proceeds as follows:

Surface of the CNTs is functionalized with some chemical receptor and the drug molecules are encapsulated through the open end which is capped with some biodegradable chemically removable cap. Degradation may be sensitive to the environment such as pH, or may be initiated by an external source. Nanocapsule is then introduced into the body by intravenous injection or orally, whereby it locates to the target site through the use of the chemical receptors. For example often cancer tumours over express folate receptors and thus the nanocapsule selectively bind to these cells. The cell ingests the nanocapsule, for example by receptor-mediated endocytosis. The chemically removable cap is triggered and either falls off or biodegrades and finally the nanotube spill its contents into the cell and thus the drug is delivered.

Ideally nanotube carrier should be engineered such that when outside the body it is energetically favourable for the drug molecule to be encapsulated, and once inside the desired cell it is energetically favourable to be ejected, and thus depositing the desired drug at the target site.

Drug delivery with CNTs

Numerous reports have been published on the *in vitro* efficacy of the drug delivery with SWNTs. For improving the pharmacological properties of many drugs, the development of CNTs as drug delivery system is of great importance.

Murakami investigated the adsorption and intracellular release of anti-inflammatory glucocorticoid dexamethasone (DEX) onto the surface of oxidated CNHs. Venkatesan studied the adsorption of erythropoietin (EPO) in a variety of porous carbon nanomaterials, including CNTs. *In vivo* results indicated that CNTs show the highest performance and biological effect in the presence of an appropriate surfactant. Bianco functionalized MWNTs with fluorescein (FITC) and antibiotic amphotericin B (AmB), AmB with its antifungal activity retained was delivered into the cells effectively. Thereafter, anticancer drugs methotrexate (MTX) was also translocated into the cells with MWNTs by the same method. Yu and co-workers synthesized gonadotropin releasing hormone (GnRH)-MWNTs complexes and verified their anticancer effect to the prostate cancer cells. Feazell's study shows that a nontoxic platinum (IV) complex could be covalently conjugated to the surface of SWNTs and delivered into the cells, which released toxic anticancer drug cisplatin by means of the reduction of Pt^{4+} at low pH value within cells. Its delivery efficiency was 6–8 times that of cisplatin. In another study, modified platinum complex with folic acid and CNTs killed the cancer cells selectively, with delivery efficiency two orders of magnitude higher than that of platinum complexes.

Nanoprecipitation was also used to load anticancer drug into the interior of CNHs and its anticancer effect was 4–6 times that of cisplatin. Chen realized the delivery of paclitaxel toxoid (Taxoid) into the cancer cells specifically with biotin-SWNTs and the intracellular release due to cleavable linking. As the large hydrophobic surface, functionalized carbon nanotubes are able to interact with aromatic molecules by π-stacking supermolecular interaction. In Liu's study, anticancer drug doxorubicin (DOX) was conjugated to SWNTs by covalent bending and noncovalent bonding, respectively. CNTs showed higher loading capacity than the conventional liposomes. The loaded DOX was released in acidic environment, while it's loading and release behavior was relevant to the diameter of CNTs. After functionalized with cyclic arginine-glycine-aspartic acid (RGD) peptide as targeted moiety, the complex showed relatively high specific delivery and destruction efficiency to the RGD receptors-positive cells. Similar results obtained by Ali-Boucetta show MWNTs are able to enhance the destruction efficiency of DOX to cancer cells.[35]

Delivery of chemotherapeutic agents

In case of cancer treatment, proper amounts of drugs need to be directed to the targeted tissue with minimum unwanted effects of the drugs on normal, healthy tissue.

Enhanced permeability and retention (EPR) effect is one of the strategies to deliver drugs specifically to a tumour site. The EPR effect is due to leaky vascular structures and an impaired drainage system of tumours. Blood vessels in tumour tissues have pores with size from 100–800 nm while in case of healthy tissues blood vessels have pores from 2 to 6 nm. Therefore, nanoparticles of 100 to 700nm penetrate selectively through the pores of tumour blood vessels and preferably accumulate at the tumour site. By conjugating SWCNTs with a therapeutic agent, whole conjugate size can be designed to be from 100 to 700 nm. In such an attempt, SWCNs were conjugated with paclitaxel (PTX) and showed high efficacy in inhibiting tumour growth in mice. SWCNTs were first functionalized with branched poly (ethylene glycol) (PEG) chains; PTX was then linked to the "termini" of branched PEG via amide bonds. In this study, PEG chains were chosen to functionalize SWCNTs because it was shown previously that intravenous injection of SWCNTs coated with PEG into mice did not cause any toxic effects over several months. The SWCNT–PTX conjugates were shown to stay in the blood for longer times (81.4±7.4 min) than Taxol (the clinical drug formulation of PTX) (18.8±1.5 min) and PTX coated with PEG (22.8±1.0 min). This prolonged circulation time together with EPR effects allowed for much higher accumulation of the drug at tumour

sites (10-fold higher than Taxol and 6-fold higher than PEG–PTX after 2 h of injection and 6- and 4-fold higher, respectively, after 24 h of injection).[36] The high delivery efficacy of PTX by SWCNT subsequently resulted in tumour inhibition of SWNT–PTX (5 mg/kg PTX) for the 4T1 tumour model which is known to be resistant to PTX treatments.

CNTs can also be conjugated with a ligand that binds specifically to receptors that are over-expressed on cancer cells. Active targeting method has been used to deliver drugs attached to CNT–ligand conjugates to tumour cells. PEG coated- SWCNTs were linked with an arginine-glycine-aspartic acid (RGD) peptide for the specific delivery of the conjugate to integrin αvβ3-positive tumours in mice via ligand–integrin binding. The SWCNT–PEG–RGD demonstrated high tumour uptake of approximately 10–15% of the injected dose (ID) per gram, a significant increase from approximately 3–4% ID per gram for SWCNT–PEG without RGD.[37] CNTs can also be used for hyperthermia treatment of cancer.

In one study C. Tripisciano et al. encapsulated cisplatin (Cis-Diamminedichloroplatinum (CDDP) – a platinum-based chemotherapy drug) into SWCNT which discharged 68% of molecule from tubes after 72 h of immersion in physiological solution. After the encapsulation procedure and the release examination, the impact of the as produced system was tested on prostate cancer cell lines (DU145 and PC3). The results obtained for the PC3 cell line show that the highest CDDP-SWCNTs concentration affected the viability as strongly as the highest free CDDP concentration since the concentration of the anticancer agent is approximately the same. For the DU145 cell line the highest CDDP-CNTs solution caused a less toxic effect than the same amount of free CDDP.[38]

Peptide delivery

Application of CNT as a template for presenting bioactive peptides to the immune system has been studies by Pantarotto et al.[39] For this purpose, a B-cell epitope of the foot and mouth disease virus (FMDV) was covalently attached to the amine groups present on CNT, using a bifunctional linker. The peptides around the CNT adopt the appropriate secondary structure for recognition by specific monoclonal and polyclonal antibodies. The immunogenic features of peptide–CNT conjugates were subsequently assessed *in vivo*. Immunisation of mice with FMDV peptide–nanotube conjugates elicited high antibody responses as compared with the free peptide. These antibodies were peptide-specific since antibodies against CNT were not detected. In addition, the antibodies displayed virus-neutralising ability.

Salvador-Morales et al.[40] showed that pristine CNT activate the complement following both the classical and the alternative way by selective adsorption of some of its proteins. Because complement activation is also involved in immune response to antigens, this might support the enhancement of antibody response following immunisation with peptide–CNT conjugates.

Protein delivery

Carbon nanotubes have been applied as a new class of potential molecular transporters for protein delivery both *in vitro* and *in vivo*.

Strepavidin (SA), a protein with clinical applications in anticancer therapies was conjugated to biotin functionalized SWNTs, which was also fluorescently labeled with 5-(5-aminopentyl)thioureidyl fluorescein to visualize the cellular uptake of nanotubes by human promyelocytic leukemia (HL60) cells and human T cells (Jurkat). It is indicated that SA is delivered into cells and exhibited toxicity to HL60 cells. The authors proposed that SWNTs nonspecifically associate with hydrophobic regions of the cell surface and internalize by endocytosis. Various proteins such as alexa-fluor bovine serum albumin (BSA, 66 kDa), protein A (SpA 42 kDa), cytochrome c (12 kDa), and fluorescein isothiocyanate-labeled human immunoglobin G (hIgG, 150 kDa) were also adsorbed spontaneously on the sidewalls of acid-oxidized SWNTs due to hydrophobic interactions and can be readily transported inside various mammalian cells.[41] It has been proposed that the nanotubes acted as the transporter *via* the energy dependent endocytosis pathway or energy independent nonendocytotic pathway.

si-RNA delivery

A siRNA, known to silence the gene encoding lamin A/C protein present inside the nuclear lamina of cells, has been attached to SWNTs functionalized with phospholipids *via* cleavable disulfide linkage. This siRNA delivery system exhibited a 2-fold advantage over transfection by lipofectamine with the same 500 nM siRNA concentration.[42] Another CXCR4 siRNA has also been conjugated to the same phospholipids functionalized SWNTs. It was found that SWNTs could be used as molecular transporters for human T cells and primary cells and deliver superior silencing effects over conventional liposome-based nonviral agents. Chemically functionalized SWNTs with hexamethylenediamine (HMDA) and poly (diallyldimethylammonium) chloride (PDDA) can bind with negatively charged ERK1 and ERK2 siRNAs by electrostatic interactions. PDDA–HMDA–SWNTs exhibited less cytotoxicity than the liposomal transfection reagent on isolated rat heart cells at concentrations up to 10 mg/L. PDDA–HMDA–SWNTs loaded with extracellular signal-regulated kinase (ERK) siRNA were able to suppress the expression of the ERK target proteins in primary cardiomyocytes by about 75%.

Nucleic acid delivery

Nucleic acids play an important role in biomedicine. Nearly 2000 kinds of hereditary diseases, as well as tumour occurrence, viral infection, and radiation to human body are found to be associated with DNA structure. Nucleic acids can conjugate to the surface of CNTs by covalent bonding and then hybridize selectively with complementary sequences.

Functionalized carbon nanotubes are able to interact with plasmid DNA through the electrostatic interaction and penetrate cell membranes with low cytotoxicity. The amount to bind DNA is function of the surface area and charge density of CNTs. Transfection of the plasmid DNA could be achieved effectively with nickel-embedded carbon nanotubes in magnetic field with a transfection efficiency equivalent to that of viral vector approach. Biocompatibility of this so-called nanospearing technique is fairly high.

In most cases, the immobilization of DNA onto CNTs is achieved by electrostatic interaction, which could only provide a metastable complex for gene transfection, and the efficiency is determined by the chemical groups on the surface of CNTs. The application of polymers such as polyethylenimine (PEI), polyamidoamine (PAMAM) dendrimers can solve this problem. Liu immobilized DNA on the surface of MWNTs firmly with PEI, and its transfection efficiency was three times higher than that of PEI and four orders of magnitude higher than that of DNA alone. Pan investigated the delivery of Survivin antisense oligonucleotide (ASOND) into the hepatic cancer cells mediated by CNTs-PMAMA dendrimer and its effect on liver cancer cells. They found that the compound can serve as an efficient gene vector with high inhibition to the proliferation of cancer cells.[43]

Delivery of genes

Gene therapy is a method to use a gene to promote cells to produce their own therapeutic proteins. Methods to deliver genes to cells can be divided into two main categories: viral gene delivery and non-viral gene delivery. In viral gene delivery, the genes are carried in the viral DNA into the cells due to the ability of viruses to enter the cells. The advantages of this method include high delivery efficacy and high levels of gene expression. However, several disadvantages such as immunogenic and inflammation responses of host tissue have restricted the applications of viral vectors in human use. On the other hand, non-viral delivery of genes to cells uses means other than viruses to transport genes inside the cells. The methods used in non-viral vectors can be either physical or chemical. Physical forces can be used to "push" genes into cells. Chemical means use other materials (such as lipids, polymers or proteins) to conjugate with genes and then direct them to the cells. One of the challenges for non-viral gene delivery is to achieve high gene transfer efficiency. There are several barriers for delivering DNA to the targeted cell population, internalizing DNA by the cells and transporting the DNA to desired cellular compartments. Those barriers include physical

barriers (including biological membranes, such as the endothelium, the plasma membranes, and the nuclear membranes) and chemical barriers (including extracellular, endosomal/ lysosomal, and cytosolic degradation pathways). Currently, microinjection (that is, using micro-needle-injected DNA) is a common method that can bypass both physical and chemical barriers to deliver DNA to cells. However, the microinjection method has disadvantages of having to manipulate cells one at a time under a microscope and micron-sized tip radii which cause damage to cell membranes. Recently, vertically aligned CNFs (VACNFs) grown on flat substrates have been employed for delivering DNA to cells. These VACNFs were coated with DNA and then pressed onto cellular matrices to deliver coated DNA to the cells (Chinese hamster ovary cells).

The advantages of this method include its minimal cell membrane disruption and its ability to simultaneously deliver DNA to a large number of cells. Multiple cells were penetrated by the VACNF array and cells continued to proliferate after 48 h. More importantly, colonies of cells expressing the delivered DNA containing an enhanced green fluorescent protein (eGFP) were observed after 22 days. Another advantage of using VACNF arrays for DNA delivery is that the arrays can be indexed and retained and assimilated by the cells, thereby allows one to track the delivery of genes and proliferation of cells. In another report by McKnight et al., researchers used spatially indexed VACNF arrays and were able to track the development of the cells and expression of the delivered DNA.[44]

Delivery of vaccines

Efforts are continuing to develop novel systems for the delivery of protective antigens. The basic idea of using CNTs in vaccine delivery involves linking an antigene to CNTs, without losing its conformation, thereby inducing an antibody response with the right specificity. However, it is equally important that the incorporated CNTs do not possess intrinsic immunogenicity and, hence, trigger an immune response. CNTs therefore act as templates, upon which chiral molecules are attached, which in turn act as centres for molecular recognition. Peptides derived from VP1 protein of the foot-and-mouth-disease virus (FMDV) were coupled to SWNTs. Serum samples from inoculated, Balb/c mice were collected and analyzed by enzyme-linked immunosorbent assay for presence of antipeptide antibodies. Peptide-CNT complexes were shown to elicit greater immune response against the peptides, with no detectable cross-reactivity to the CNTs, confirming the no immunogenicity of the carrier. It was also observed that the CNT protein complex enhanced the immune response when attached to an antigen, which strengthens the possibility of incorporating CNTs in vaccines.

Bianco et al demonstrated that the presence of cationic f- CNTs in the delivery of synthetic oligodeoxynucleotides containing CpG motifs (ODN-CpGs) improve the immunostimulatory properties of ODN-CpGs *in vitro*. Synthetic oligonucleotides containing CpGs are reported to confer nonspecific protection against cellular pathogens and enhance antigen-specific immune responses. These properties have made them target candidates for incorporation in vaccines. To evaluate the immunostimulatory properties of CNTs, various ratios of CNT-ODN CpG complexes were incubated with splenocytes. The efficiency of the process was measured by the amount of interleukin-6 (IL-6), a proinflammatory cytokine whose production is stimulated by ODN-CpG secretion in the supernatant of the culture. The results showed higher levels of IL-6 when f-CNTs were in complex with ODN-CpGs than when only ODN-CpGs were incubated with splenocytes.[45]

CNTs in regenerative medicine

(1) Bone regeneration

One of the first studies using carbon nanofibres (CNFs) in scaffolds for tissue regeneration was the study by Price et al. In this *in vitro* study, researchers dispersed CNFs in polycarbonate urethane (PCU) to create composites and tested it on adhesion of osteoblasts, fibroblasts, chondrocytes, and smooth muscle cells on the composite scaffolds. They found that the composites with smaller scale carbon fibers promoted osteoblast adhesion but did not promote the adhesion of other cells. More interestingly, smooth muscle cell, fibroblast, and chondrocyte adhesion decreased when carbon nanofiber surface energy increased. Surface energy is an important parameter that influences cell adhesion and therefore subsequent cell functions. Price et al. also reported that greater weight percentages of high surface energy carbon nanofibers in the PCU/CNF composite increased osteoblast adhesion while at the same time decreased fibroblast adhesion.

A material that can promote osteoblast adhesion and, at the same time, decrease competitive cell adhesion is desirable in orthopaedic implants since that material can lead to faster integration of the bone to the implant surface *in vivo*.[46] This study demonstrated the versatility of using CNFs to tailor the surface structure, surface chemistry and/or surface energy of a scaffold to selectively promote the adhesion of one type of cell but inhibit functions of other types of cells. In orthopedic applications, early cellular events are critical since subsequent cell functions are influenced by such early events. Therefore an implant that can enhance osteoblast adhesion and such desired features should be designed by altering CNF content in the composite. This would lead to increase synthesis of intracellular proteins, alkaline phosphatase activity and deposition of calcium-containing mineral to make bone. This goal was achieved with the use of CNFs in a scaffold. In one experiment, carbon fiber compacts were fabricated to possess either nanophase (i.e., dimensions 100 nm or less) or conventional (i.e., dimensions larger than 100 nm) fibers and the long term cellular functions on both types of compacts were compared. Osteoblasts were cultured on the nanophase compacts and conventional compacts. The results showed that osteoblasts synthesized more alkaline phosphatise and deposited more extracellular calcium on the nanophase compacts than on the conventional compacts. Interestingly, among the nanophase compacts, calcium content in the extracellular matrix increased with decreased carbon fiber diameter. Clearly, the size of the CNFs plays an important role in increasing osteoblast functions.

(2) Neural Tissue Regeneration

Mattson et al. provided the first evidence that MWNTs can be used to support neuronal cell attachment and growth. Studies have shown that CNTs chemically functionalized with various bioactive molecules can improve neural regeneration activity including neurite branching, outgrowth and attachment of growth cones. 18–19 wt % SWCNT with polyethyleneimine (PEI) copolymer has been synthesized to effectively lengthen neurites and increase neurite branches approximately comparable to those on polyethyleneimine. Matsumoto et al. demonstrated that MWCNTs can regulate and promote neurite outgrowth when covalently bonded with neurotrophin. In addition, the high electrical conductivity of CNTs can enhance neuronal circuit network activities. Lovat et al. reported that the electrical signal transfer on neuronal networks can potentially be improved by using purified MWCNTs. In the study, CNT substrates significantly increased hippocampal neuron spontaneous synaptic currents and spontaneous firing activity compared to control substrates.

Various patterned CNT islands, matrices and films have been fabricated in order to improve neural applications. Gabay et al. microfabricated patterned substrates composed of CNT islands. After culturing neuronal cells from the cortices of one-day-old Charles River rats on the CNT island substrates, a well organized neural network was developed. It is also proven that SWNT multilayer films not only preserved the electrophysiological properties of neurons themselves but it also electrically stimulated neuronal cells for repairing damaged nerves. Jan et al. reported that the layer-by-layer assembled SWNT/polyelectrolyte composites improved the differentiation of mouse embryonic neural stem cells into neurons as well as astrocytes and helped neurite outgrowth. After 7 days of culture, the composites selectively promoted more neurons and fewer astrocytes than a common poly-L-ornithine substrate used for neural studies. The results indicated that CNT composites were not only cytocompatible for stem cell growth, but also contributed to differentiating stem cells to neuronal cells.[47, 48]

Toxicity

Graphite has been associated with increased dermatitis and keratosis. Thus investigation is needed to determine the potential danger of exposure to CNT. Studies so far have focused on the effects of pristine CNT on different cell lines like human epidermal keratinocytes, HEK293 human embryonic kidney cells, human acute monocytic leukemia cell lines, human T cells and alveolar macrophages. When SWNT exposure was studied on keratinocytes, oxidative stress and cellular toxicity were detected from the presence of free radicals and peroxides, leading to antioxidant depletion and loss of cell viability.[49] Inflammatory responses were also reported on the same cell line by MWNT. The mechanism is likely to be due to the production of reactive oxygen species, leading to the activation of the nuclear transcription factor-nB. Moreover, CNT can activate the human serum complement system via the classical and the alternative pathway, generating proinflammatory peptides. Studies on the biocompatibility of SWNT on HEK293 human embryonic kidney cells showed a cell growth inhibition via induction of apoptosis and decrease of cell adhesion ability. Pristine nanotubes usually contain transition metal catalysts like iron or nickel, which could explain the generation of free radicals, however, in the case of the MWNT no iron was detected before cellular interaction.

A comparative study on the toxicity of pristine and oxidized MWNT onto human Jurkat T leukemia cells has shown that the latter were more toxic. CNT tend to aggregate in ropes, decreasing in aqueous solubility and increasing in cytotoxicity. The type of nanotubes used in such studies is therefore extremely important. Comparison of cytotoxicity induced by different carbon materials in alveolar macrophages showed that SWNT provoke the highest toxic effect, followed by MWNT, quartz and fullerenes.

A study on the effect of the CNT length on cytotoxicity showed that the inflammatory response was higher for the CNT of 825 nm in comparison to that of 220 nm.[50] It has been previously shown that cytotoxicity of water-soluble fullerene derivatives is improved as the degree of surface modification is increased. Thus, it is reasonable to anticipate that functionalized and water-soluble nanotubes will be less cytotoxic. Different types of functionalized, soluble CNT have already been studied in various laboratories reporting no significant cell damage by using SWNT-RNA polymer hybrids with a concentration up to 1 mg/mL with MCF7 breast cancer cells; 90% of fibroblast survival following incubation with 5 AM fluorescein functionalized SWNT; and no toxicity for CHO and 3T3 cells interacting with protein-functionalized SWNT.

Another important aspect that plays a critical role in determining CNT toxicity is related to their bioavailability and deposition in lungs following inhalation. Lam et al. described a dose-dependent formation of epitheloid granulomas and some interstitial inflammations.[51] This study showed that nanotubes were much more toxic than carbon black and quartz. Warheit and co-workers have determined that SWNT exposure in rats produced dose-independent series of multifocal granulomas.[52] 125I-labelled water-soluble SWNT were radiotraced in mice following intraperitoneal administration. They were shown to be distributed among different tissues. Their excretion was mainly through urine, and bone accumulation was reported after 18 days without any concomitant toxicity.

In summary, pristine CNT have been found to be cytotoxic to various mammalian cell lines *in vitro* and to the skin and lungs *in vivo*. However, pristine nanotubes tend to aggregate due to their insolubility, therefore such toxicological responses are not surprising. Significant efforts have been made to improve the nanotube solubility and a remarkable reduction on toxicity has been reported using such functionalized CNT. The toxicological profile of CNT depends on many parameters such as the type of nanotubes, the presence of impurities, the length of the tubes, the type of functionalization and the molecular nature of the conjugated groups. More systematic *in vitro* and *in vivo* investigations using biologically compatible CNT are necessary to establish a general toxicological profile of CNTs.

CONCLUSION

Carbon nanotubes play an important role in nanomaterial research due to their mechanical, optical, electrical and structural properties. Many studies have clearly shown that carbon nanotubes, following appropriate surface modification to render them biocompatible, hold great promise as nanovectors for the delivery of a variety of therapeutic and diagnostic agents. The chemistry of CNT offers the possibility of introducing more than one function on the same tube, so that targeting molecules, contrast agents, drugs, or reporter molecules can be used at the same time. Carbon nanotubes today represent a class of emerging nanovectors that are capable to intracellularly deliver biologically functional peptides, proteins, nucleic acids and small molecules covalently or noncovalently attached on their surface.

Future studies will determine the opportunities as well as the limitations that these novel nanovectors hold towards their clinical realization. The characteristic one-dimensional structure of carbon nanotubes makes them an ideal platform in biomedical application, including drug delivery, molecular imaging, and gene therapy. The exterior surfaces of CNTs can adsorb several molecules and bind various chemical groups for solubilising and targeting, whereas the interior cavity is able to encapsulate small molecules and ions. Moreover, CNTs can penetrate the cells with minimal cytotoxicity and thus these serve to be promising carriers for delivery of drugs.

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