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Review

# Delivery of drugs and biomolecules using carbon nanotubes

*Sandeep Kumar Vashist* [a,b], *Dan Zheng* [a,c], *Giorgia Pastorin* [d], *Khalid Al-Rubeaan* [e], *John H.T. Luong* [f,g], *Fwu-Shan Sheu* [a,b,*]

[a] *NUSNNI-NanoCore, National University of Singapore, T-Lab Level 11, 5A Engineering Drive 1, Singapore 117580, Singapore*
[b] *Department of Electrical and Computer Engineering, National University of Singapore, Engineering Drive 1, Singapore 117576, Singapore*
[c] *Department of Chemistry, National University of Singapore, 3 Science Drive 3, Singapore 117543, Singapore*
[d] *Department of Pharmacy, National University of Singapore, S4 Science Drive 4, Singapore 117543, Singapore*
[e] *University Diabetes Center, King Saud University, P.O. Box 18397, Riyadh 11415, Saudi Arabia*
[f] *Biotechnology Research Institute, National Research Council Canada, Montreal, Quebec, Canada H4P 2R2*
[g] *Department of Chemistry, University College Cork, Cork, Ireland*

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ABSTRACT

Carbon nanotubes (CNTs) have emerged as one of the most advanced nanovectors for the highly efficient delivery of drugs and biomolecules. They offer several appealing features such as large surface areas with well defined physico-chemical properties as well as unique optical and electrical properties. They can be conjugated non-covalently or covalently with drugs, biomolecules and nanoparticles. Albeit some pending concerns about their toxicity *in vitro* and *in vivo*, functionalized CNTs appear to exhibit very low toxicity and are not immunogenic. Thus, they could be promising carriers with a great potential for the development of a new-generation delivery system for drugs and biomolecules. There have been significant advances in the field of CNT-based drug delivery, especially in the specific targeting of anticancer and anti-inflammatory drugs for tissues and organs in the body, where their therapeutic effect is highly required. Other promising applications are the delivery of DNA, RNA and proteins.



## Contents



* *Corresponding author at:* Department of Electrical and Computer Engineering, National University of Singapore, Engineering Drive 1, Singapore 117576, Singapore. Fax: +65 68725563.
E-mail address: elesfs@nus.edu.sg (F.-S. Sheu).
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## 1. Introduction

Simple or sophisticated drug delivery systems (DDS) are engineered to improve the pharmacological profile of bioactive molecules while preserving them from deactivation throughout the body. Without DDS, the efficacy of a drug relies entirely on its physico-chemical properties and ability to reach a target site where its activity is necessary. Conversely, DDS have opened new possibilities due to their ability to protect the molecule of interest and selectively target specific compartments without adversely affecting the surrounding tissues. Current DDS models are mainly liposomes, dendrimers, polymers, virus-based systems, cyclodextrins, nanoparticles, fullerenes and nanotubes (Table 1). There is also a critical need to build versatile platforms, which can specifically target, efficiently deliver and proficiently visualize the site of actions of these multifunctional conjugates. To date, such platforms using nanoscale materials are emerging for drug release and imaging, e.g. nanoshells, quantum dots (QD) or nanowires [1].

Carbon nanotubes (CNTs) could be one of the most advanced nanovectors for the highly efficient delivery of drugs and biomolecules owing to their large surface with unique optical and electrical properties. They can be conjugated non-covalently or covalently with drugs, biomolecules and nanoparticles towards the development of a new-generation delivery system for drugs and biomolecules. This article provides a concise review of CNT-based systems developed in the past decade for delivery of drugs and biomolecules. The toxicity of CNTs is also addressed followed by future possibilities for technology development. Although significant developments have taken place in the last decades, there are still numerous challenges, which need to be overcome to render this technology mature enough for commercialization. These challenges involve (1) the synthesis of ultrapure CNTs, bioconjugation, surface functionalization and modification strategies for the development of biocompatible functionalized CNTs; (2) thorough understanding of the mechanisms of interaction of CNT-drug/biomolecule complexes with cells, tissues and other physiological systems; (3) development of international guidelines for toxicity analysis as well as regulatory aspects and the safety issue related to the use of nanomaterials, as stated in the bioethical guidelines; and (4) increasing acceptability for the adoption of this novel material by demonstrating its advantages in terms of correlation grid analysis and potential end-user trials, where the developed technology is compared with the existing ones.

## 2. Drug delivery systems

### 2.1. *Current drug delivery systems*

Liposomes are one of the best known DDS, made up of a lipid bilayer, which mimic cells in terms of cell membrane, while their inner hollow part can be filled with one or more drug molecules. The most intriguing aspect is their excellent biocompatibility and potential use as a temperature- or pH-sensitive drug carrier. A few prototypes have been converted in formulation already in phase II and III of clinical trials. For instance, Doxil®, DaunoXome®, Caelyx® have been developed to replace conventional chemotherapy for the treatment of metastatic ovarian cancer. As the first generation of DDS, liposomes with relatively big dimensions (90–150 nm), might limit their use in nanobiotechnology (by definition below 100 nm). These systems also suffer from physical instability due to their amphiphilic nature and, in some cases, they seem responsible for superficial toxicity, a so-called “hand and foot syndrome” [2,3], most probably due to the prolonged circulation time of liposomes.

Unlike circular shaped liposomes, dendrimers are highly branched, multiple-shaped polymers with a few nanometers in diameter. The main advantage is good control of their dimensions and their vast exposed surface, optimal for facile conjugation with different molecules ranging from therapeutic agents to targeting molecules and even fluorescent dyes [4]. Although they are extremely promising in delivering molecules or nucleic acids, some dendrimer-based multifunctional systems have shown significant cytotoxicity. In other cases, functionalized dendrimers release a target drug very slowly, less than 15% over 20 h. Therefore, “smart” polymers with high sensitivity towards pH changes and reduced toxicity have been recently developed to overcome such limitations. An exception in the use of dendrimers as a drug delivery vehicle is their investigation as bioactive agents by Starpharma. This dendrimer-based microbicide (VivaGel) is

**Table 1 – Examples of drugs delivered through the most recently-investigated drug delivery systems.**

| Drugs | Nano-material | Size (in nm) | Drug loading and release | Therapeutic agents delivered | Applications | Ref. |
|---|---|---|---|---|---|---|
| Doxorubicin & Co. | Liposomes | 100–200 | Range 30–50 mg/m$^2$ | Doxorubicin (Doxil, Caelyx), Doxorubicin + Galactosamine, Daunorubicin | Cancer therapy | [19] |
| | Nano-particles | 140–250 | Drug entrapment efficiency: 82% | Doxorubicin, $IC_{50}$ values: 103 ng/mL | Cancer therapy | [20] |
| | Dendrimers and smart polymers | 2–15 | 20 molecules of Adr. at the adriamycin/dendrimer ratio of 40. Release at pH = 7.4: 0% At pH = 5.5: 80% in 24hrs | Adriamycin, $IC_{50}$ = 1.6 mM | Cancer therapy | [21] |
| Pt-based drugs (e.g. Cisplatin, Carboplatin) | Nano-particles | 250 | Concentration of Cisplatin: 37 μM. Release: 73.8 ± 5.6% through additional heating (20% without heating) | Cisplatin | Cancer therapy | [22] |
| Paclitaxel | Liposomes | 100–200 | | Paclitaxel (LipoTaxen™) | Cancer therapy | [23] |
| | Nano-particles | 140–250 | Drug entrapment efficiency: 95% | Paclitaxel, $IC_{50}$ values: 9.8 ng/mL | Cancer therapy | [20] |
| | Nanoparticles +polymers | <100 | Drug entrapment efficiency: 79.6% | Paclitaxel | Cancer therapy | [24] |
| | Nano-particles | 140–250 | Total entrapment efficiency: 85% (74% for DOX and 96% for PTX) | Paclitaxel + Doxorubicin | Cancer therapy | [20] |
| | | | PTX release: about 25% in 48 h, 60% in one week, and almost complete release over three weeks | | | [25] |
| | VIRAL particles | <100 | | Paclitaxel, Docetaxel | Cancer therapy | [26] |
| | Fullerenes | 120–145 | Doses in ranges of 0.004–0.05 μg/mL | Paclitaxel, $IC_{50}$ = 253 nM. T ½ = 80 min | Cancer therapy | [27] |
| Amphotericin B | Liposomes | 100–150 | Single dose of 1–20 mg/kg | Amphotericin B (AmBsome™ or Fungisome™). T ½ = 5–24 h | Antifungal treatment | [28] |
| | Dendrimers and block-copolymers | | 15 mg/ml | Amphotericin B | Antifungal treatment | [29] |
| | Nanoparticles (of PLGA biodegradable polymers) | 165.6 ± 2.9 | Entrapment of 34.5 ± 2.1% at 10% w/w drug loading. Biphasic release | Amphotericin B | Antifungal treatment | [30] |

effective in the prevention of HIV and sexually transmitted infections (STI).

Inorganic nanoparticles appear very promising not only as DDS but also as therapeutic and contrast agents. For example, superparamagnetic iron oxide nanoparticles [5], under the influence of an alternating field, release localized heat with a concomitant induction of apoptosis in tumoral cells through such hyperthermia. Alternatively, gold nanoparticles provide anti-angiogenic properties and anti-inflammatory activity [6]. In fact, gold sodium thiomalate (Auranofin or Ridaura®) has been approved for the treatment of inflammation associated with rheumatoid arthritis [7,8].

Viral nanoparticles, especially those incorporating adenoviruses, seem particularly suitable for gene therapy, vaccines and drug delivery, on the basis of their incomparable transfection efficiency and specificity [9]. However, their determined effects are transient and localized at the site of injection. Viral systems might also mutate rapidly, thus causing non-specific toxicity upon delivery and increasing skepticism in terms of their safe application.

Fullerenes are carbon-based materials used for their intrinsic ability to behave as antioxidant [10], antibacterial [11], contrast agent [12,13] and sensitizer for photodynamic therapy [14]. A major drawback is their accumulation in the organism mainly in the liver, due to their extensive binding to plasma proteins, thus hampering any application in nanomedicine.

Cyclodextrins (CD) are cyclic oligosaccharides containing at least several D-(+) glucopyranose units attached by α-(1, 4) glucosidic bonds. The three common CD are α-, β-, and γ-CD with 6, 7, or 8 glucose units, respectively. CD with hydrophobic inner cavities and hydrophilic outer surfaces are capable of interacting with guest molecules to form noncovalent inclusion complexes. Both cationic and anionic CD are also commercially available or can be derivatized from neutral CD. Hydroxypropyl, hydroxylethyl, sulfobutyl, and various methylated CD derivatives with very high purity are available in bulk quantities with affordable prices. The binding constant for several drug/CD complexes ranges from 0 to 100,000 $M^{-1}$ [15]. To date, several drugs are known to form inclusion complexes with neutral and charged CD. In general, charged CD form better complexation with opposite charged drugs. The application of CD and their derivatives for drug delivery, particularly in protein/peptide drug delivery and gene delivery can be found elsewhere [16]. A limiting factor for the use of CD is their ability to form an inclusion complex with a guest molecule, which in general is a small water-insoluble drug. Then, the drug must be able to partition out of the complex once it is in the conjunctival epithelium (ocular formulation) or the dermal region (topical application). Certain cyclodextrins, e.g. dimethyl β-CD cannot be used for corneal ophthalmic applications due to the sensitive nature of corneal epithelium [17].

Polymers have been well positioned in the field of drug delivery. Pharmaceutical polymers include vinyl polymers, cellulose ethers, polyesters, silicones, polysaccharides and other biopolymers. Polymers are widely used as binders in tablets to flow controlling agents in liquid, emulsion and suspension. Polymers can also be used as film coatings to enhance drug stability, modify drug release characteristics, and disguise the bitter/unpleasant taste of a drug. Swelling controlled release systems, biodegradable systems, and osmotically controlled DDS have been exploited. Other mechanisms are based on ultrasound-, temperature-, pH and electric current-responsive drug release. Detailed information for the use of polymers in drug formulation and responsive release can be found elsewhere [18].

### 2.2. *CNTs as a drug delivery system*

Since the landmark paper by Iijima in 1991 [31], CNTs have been used for diversified applications such as sensing, nanotechnology, material science, electronics, optics, gas storage and biomedicine. They have been one of the most highly researched materials of the last two decades in the 20th and 21st century. Fig. 1 shows the continuously increasing research efforts for using CNTs as a DDS during the last decade. Single-walled CNTs (SWCNTs) and multi-walled CNTs (MWCNTs), the two most dominant forms have been extensively used for the delivery of drugs and biomolecules. Most of the commercially available CNTs are produced by chemical vapor deposition (CVD). Intensive research efforts are now being pursued for developing CNTs with very high purity of >99.99%, as it has been firmly established that the presence of even trace amounts of metal impurities still affects the properties of CNTs and may contribute significantly to toxicity.

CNTs possess unique and excellent structural, optical and electrical properties for the development of advanced drug delivery systems. Their very large surface area, allows multi-conjugation of various molecules on the sidewalls. Molecules containing aromatic groups can be easily bound to CNTs non-covalently by strong $\pi$–$\pi$ interactions. 1-D functionalized CNTs (*f*-CNTs) could improve the binding to a single cell by interacting through multiple binding sites due to their flexibility [32].

The intrinsic optical and electrical properties of CNTs are specifically utilized in imaging and therapeutic applications.

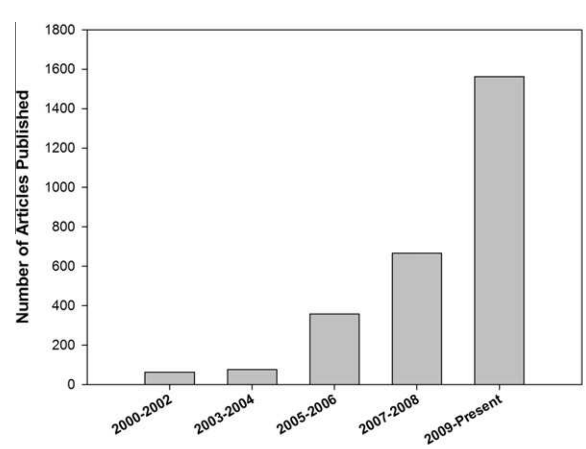


**Fig. 1 – Number of articles published in the last decade pertaining to CNT-based drug delivery applications (based on data taken from www.sciencedirect.com on Mar. 21, 2011 using “carbon nanotubes” and “drug delivery” in the advanced search option).**

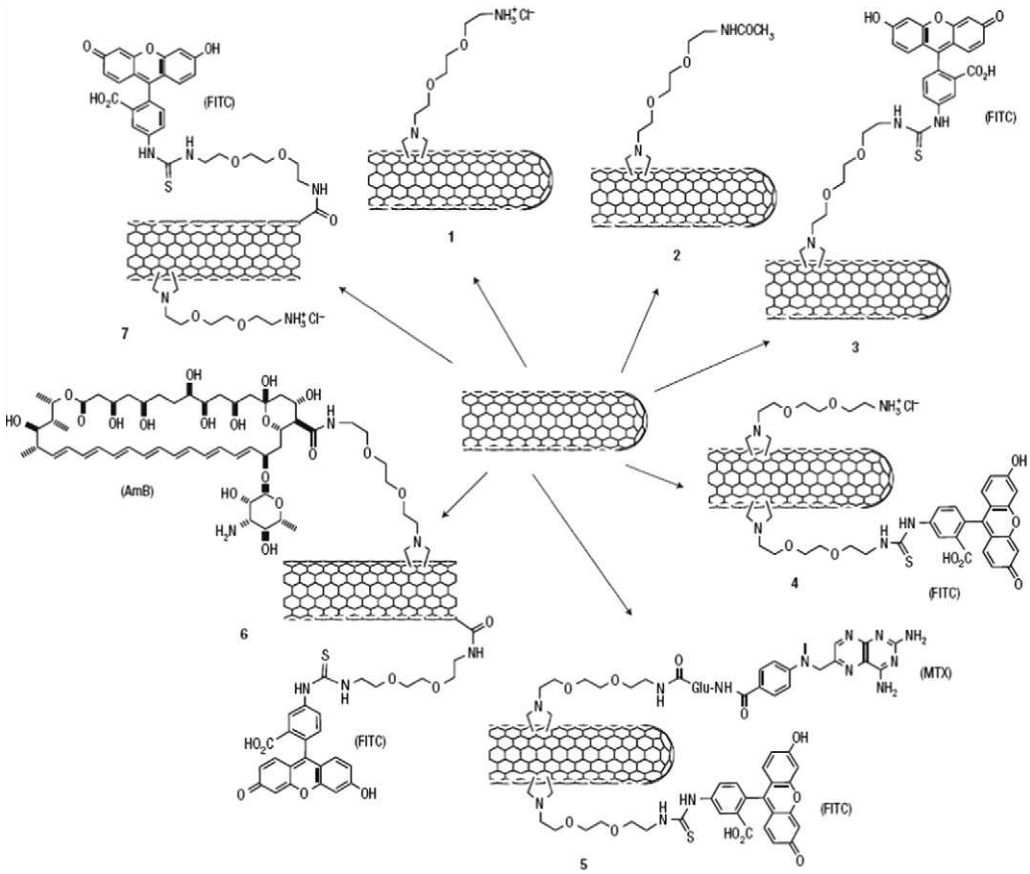

**Fig. 2 – Molecular structures of CNTs functionalized covalently with different types of small molecules [49]. Reprinted with permission from Nature Publishing Group.**

SWCNTs absorb light strongly in the near-infrared (NIR) range (800–1600 nm), which contains the tissue transparent region of electromagnetic wavelengths (800–1400 nm). Therefore, they are extensively employed in photothermal therapy [33–35] and photoacoustic imaging [36]. The optical properties of SWCNTs can also be used for Raman detection and imaging [34,37,38].

Pristine CNTs are intrinsically hydrophobic and cannot disperse uniformly in most solvents and biological media, i.e. they cannot be employed directly for drug or biomolecular delivery. Thus, functionalization must be developed for improving their biocompatibility and solubility, which allow further modification of CNTs with drugs and biomolecules. These methods include (a) non-covalent functionalization outside CNT (e.g. on external walls); (b) defect functionalization at the opened tips and sidewalls of CNT; (c) covalent functionalization (also outside CNT on their sidewalls); and, (d) encapsulation of bioactive molecules or drugs inside CNT. The most common method for non-covalent modification is to absorb functional moieties containing aromatic groups onto the external wall of CNT through $\pi$–$\pi$ interactions [39–42]. As an example, $C_2B_{10}$ carborane cages are attached to SWCNT side walls via nitrene cycloaddition, and their suitability for transporting large and heavy groups into the cells without any toxicity is evaluated [43]. The nido-$C_2B_9$ carborane and ethoxide group-functionalized (*f*)-SWCNTs are water-soluble with more boron atoms aggregated in tumors cells in comparison to blood and other organs. CNTs can also be modified on the defect sides, e.g. CNTs are often oxidized to introduce carboxylic groups, followed by amidation, esterification or formation of $COO^-NH_3^+$ salts. Thereafter, various hydrophilic or hydrophobic molecules can be bound to CNT via amide or ester linkages. Polymers can also be grafted to CNT by this method [40,43–47]. CNTs can also be covalently modified through 1,3-dipolar cycloaddition of azomethine ylides. Bioactive molecules/drugs/fluorescent probes, which are activated at the carboxylic groups, e.g. using benzotriazol-1-yloxytris(dimethylamino)phosphonium hexafluorophosphate, can successfully couple via $\alpha$ or $\gamma$ COOH to the free amino groups of these reactive *f*-CNTs to form a robust guest-CNT conjugate [48–53]. *f*-CNTs [amino-, acetylated-, fluorescein isothiocyanate (FITC)-labeled-, double-functionalized CNTs, etc.], electrostatically neutral or charged, are internalized by various species (e.g. cells including 3T6, 3T3, HeLa, Jurkat human T-lymphoma, MOD-K, human keratinocytes, A549, CHO, HEK293; or yeast like *Cryptococcus neoformans*, and *Saccharomyces cerevisiae*; or bacteria such as *Escherichia coli* strains), suggesting that different chemical procedures can be utilized to import diversified bioactive molecules [49] (Fig. 2). The encapsulation of guest molecules inside CNTs also protects them from inactivation or degradation by surrounding environments. The encapsulation of bioactive molecules and drugs and the functionalization of CNTs [54–60] have been reviewed extensively.

## 3. Delivery of drugs

### 3.1. Anticancer drugs

#### 3.1.1. Doxorubicin

As an anthracycline antibiotic, doxorubicin (DOX), functions as a DNA intercalating agent and has been widely used in treating various kinds of cancers. It is usually administered intravenously, resulting in its inefficient distribution, low selectivity, and inability to cross cellular barriers. However, these limitations pertaining to the traditional administration of DOX can be counteracted by using CNTs as a novel drug transporter, due to their capability of immobilizing therapeutic molecules on the surface or in their hollow space and transporting them through mammalian cell membranes.

Of interest is the development of an anticancer DDS by combining DOX, monoclonal antibody and fluorescein on the oxidized SWCNT sidewall [61] (Fig. 3). The monoclonal antibody recognizes the tumor marker, i.e. carcinoembryonic antigen (CEA) and assists in the effective binding of DOX to the desired target sites on cancer cells. The delivery of drug-SWCNT complexes to WiDr colon cancer cells results in a complete penetration into cancer cells, followed by the release of DOX to the nucleus whereas SWCNTs remain in the cytoplasm.

In another approach, DOX can be loaded on the polysaccharide materials [sodium alginate (ALG) and CHI] coated carboxyl functionalized SWCNT [62]. DOX binds to CNT at pH 7.4 and gets released at lower pH, which is a characteristic of lisosomes and certain tumor environments. Folic acid (FA) modified SWCNTs improve the selectivity of DOX release to the lisosomes of HeLa cells in comparison to DOX per se, because the folic acid receptor tends to be overexpressed on the surface of cancer cells. The use of ALG also facilitates DOX loading, while the use of CHI improves the binding of FA. There is

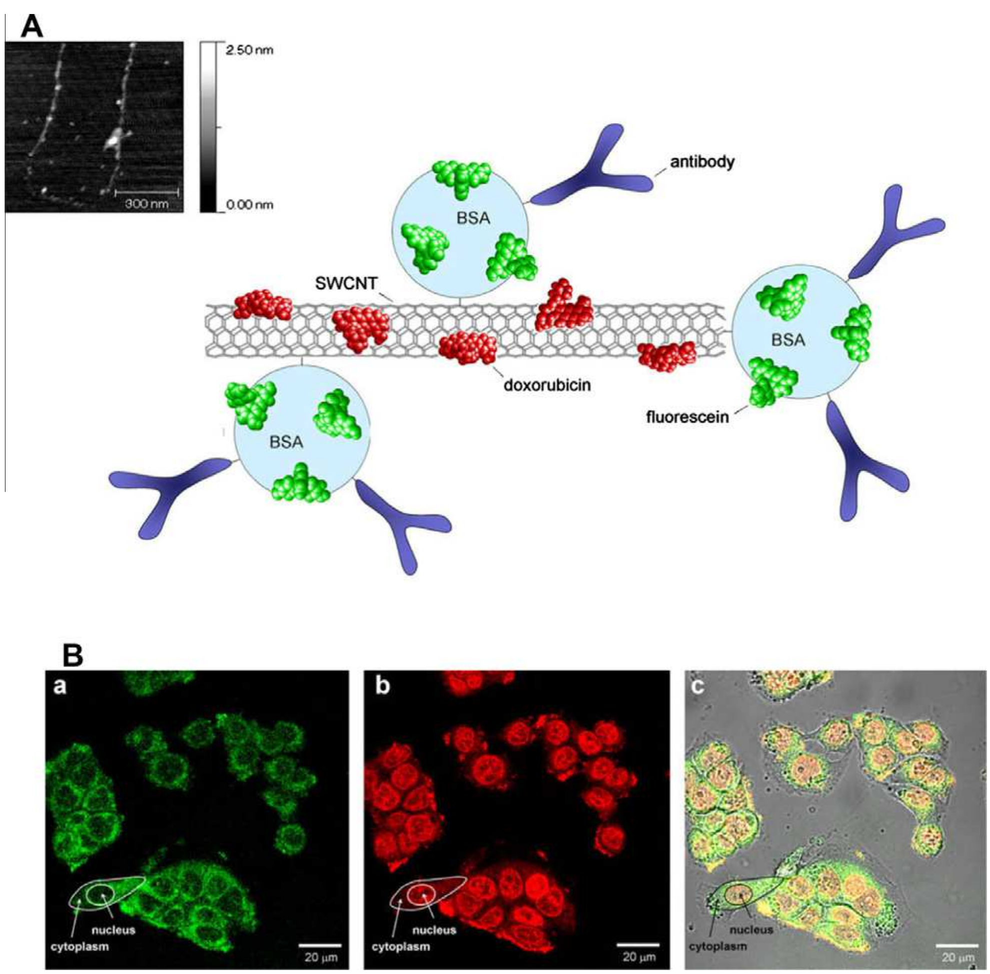


**Fig. 3 – (A) Schematic illustration of DOX-fluorescein-BSA-antibody-SWCNT complexes (red = DOX, green = fluorescein, light blue = BSA, dark blue = antibodies). Insert: AFM image of DOX-fluorescein-BSA-SWCNT complexes (without antibodies). (B) Confocal image of WiDr cells incubated with DOX-fluorescein-BSA-SWCNT complexes (a = emission measured at 500–530 nm (fluorescein), b = emission measured at 650–710 nm (DOX), and c = transmitted light image showing all channels) [61]. Reprinted with permission from Elsevier. (For interpretation of the references in colour in this figure legend, the reader is referred to the web version of this article.)**

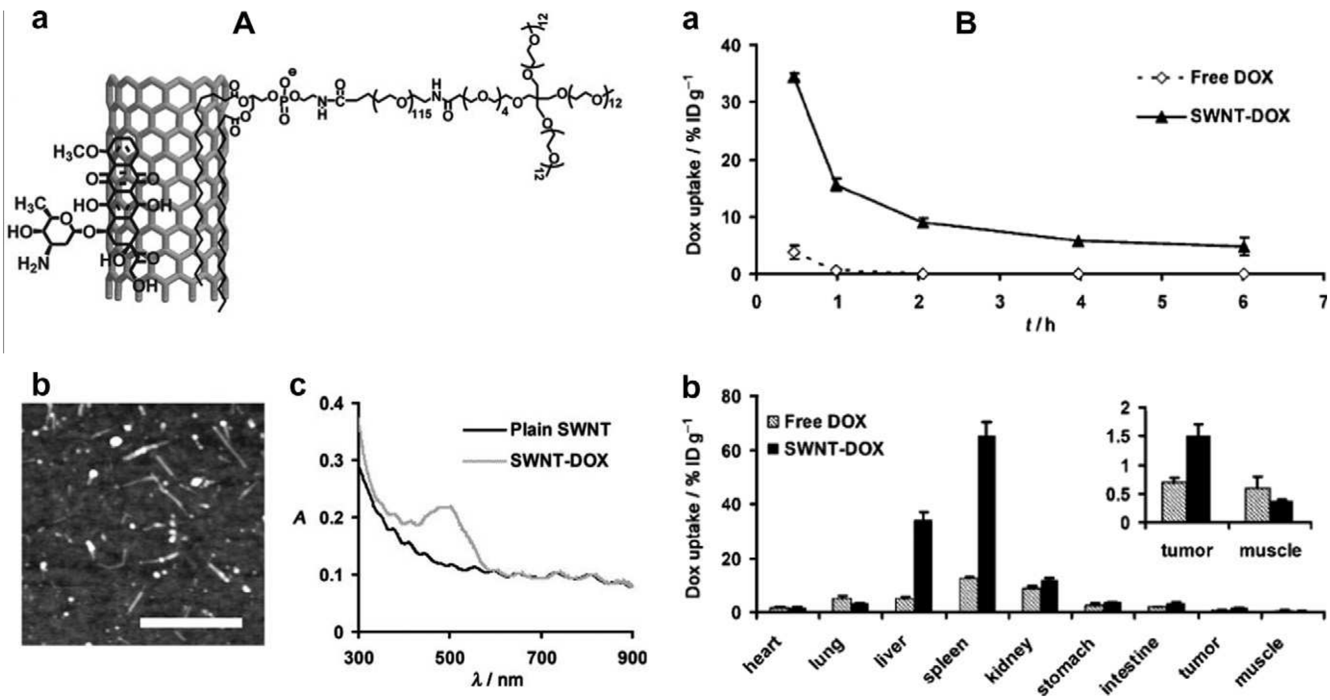


**Fig. 4 – (A) Representation (a) and AFM image (b) of the SWCNT–DOX complex. (c) UV/Vis/NIR spectra of plain SWCNTs and SWCNT–DOX. (B) Fluorescence spectroscopy study of the pharmacokinetics and biodistribution of two DOX formulations. (a) SWCNT–DOX showed prolonged blood circulation compared with free DOX. Concentrations of DOX in blood from mice treated with free DOX and SWCNT–DOX were measured by fluorescence spectroscopy at different time points after injection. (b) SWCNT-DOX had higher tumor-specific uptake and RES uptake than free DOX. Biodistribution of DOX in major organs of mice was measured 6 h after injection of free DOX and SWCNT–DOX [63]. Reprinted with permission from Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim.**

an effective release of DOX, which enters the nucleus of cancer cells and induces cell death.

High degrees of $\pi$-stacking of DOX with an ultrahigh loading capacity is attached onto the water-soluble SWCNT, which are noncovalently functionalized by phospholipid-poly(ethylene glycol) (PL-PEG5000-$NH_2$) or covalently modified by PEGylation of carboxylic groups on SWCNT [39]. The binding and release of drugs is controlled by the change in pH. The strength of $\pi$-stacking of drugs is affected by the diameter of SWCNT. The cyclic arginine–glycine–aspartic acid (RGD) peptide conjugated to soluble SWCNT acts as a ligand to impart recognition moieties for integrin $\alpha_v\beta_3$ receptors and enhances drug delivery to integrin $\alpha_v\beta_3$-positive U87MG cells. However, there is no noticeable improvement in the delivery of RGD–SWCNT–DOX when integrin $\alpha_v\beta_3$-negative MCF-7 cells are used.

The supramolecular stacking of DOX on SWCNT for *in vivo* lymphoma therapy has been studied [63] (Fig. 4). DOX is loaded on PEGylated SWCNT by supramolecular $\pi$–$\pi$ stacking. The *in vivo* pharmacokinetics profiles, biodistribution, therapeutic efficacy and toxicity of this drug delivery system are then investigated. Mice treated with either free drug or drug complexed with CNT show that SWCNT–DOX is more effective and less toxic in comparison to equimolar amounts of DOX.

A DOX–MWCNT supramolecular complex dispersed in Pluronic F127 was used to study the *in vitro* cytotoxicity of the complex in MCF7 human breast cancer cells [64]. The noncovalent DOX–MWCNT complex has improved cytotoxicity in comparison to free DOX and DOX-Pluronic F127 complex.

Amphiphilic polymers can be used to increase the solubility and anti-biofouling of CNTs as they have hydrophobic groups for attachment to the walls of CNTs, PEG for blocking protein absorption and carboxylic groups to facilitate the binding of DOX [65]. These *f*-CNTs exhibit improved solubility, greater anti-biofouling and high drug loading capability. DOX released from such *f*-CNTs acts specifically against B16F10 melanoma cells *in vitro*.

A nanocomposite composed of MWCNT difunctionalized with folate and iron (FA-MWCNT@Fe) was used as dual-targeted drug carrier for DOX delivery under an external magnetic field [66]. The FA-MWCNT@Fe has sufficient loading capacity and prolonged DOX release. It has sixfold better delivery efficiency towards HeLa cells than the free DOX due to the biological (active) and magnetic (passive) targeting of difunctionalized CNT.

Epirubicin (EPI) is a highly efficient antineoplastic in the family of doxorubicin hydrochloride. However, it causes cardiac toxicity and severe suppression of hematopoiesis. The use of CNTs as a drug carrier for EPI changes the distribution of EPI and enhances its effective concentration at the tumorous site. Therefore, EPI–CNT can be effectively employed in the treatment of tumors. CNTs form a supramolecular structure with EPI through $\pi$–$\pi$ stacking [67]. The acid-treated MWCNTs (c-MWCNTs) have higher EPI loading efficiency than the untreated CNTs. The amount of EPI release from c-

MWCNTs in the acidic medium is 1.5-folds larger than that in the netural medium.

The PEGylated MWCNTs have been reported as a drug carrier to overcome multidrug resistance (MDR) [68]. The MDR tumor cells were developed in a medium containing higher concentration of DOX. The PEGylated MWCNTs can target and accumulate in MDR tumor cells as efficiently as in non-MDR tumor cells, while the MDR cells cannot remove intracellular MWCNTs.

### 3.1.2. *Platinum-based anticancer drugs*

Cisplatin (CDDP) is platinum (Pt)-based anticancer drug commonly used to treat various types of cancers. It binds to DNA *in vivo* to induce DNA crosslinking and triggers apoptosis. However, it has a number of undesirable side-effects that limit its application. CNT-based DDS can counteract these side-effects by protecting the light sensitive CDDP from the external reactive species.

CDDP can be encapsulated inside tip-opened and shortened SWCNTs, which are treated with strong acid and annealed in a high vacuum environment [69]. SWCNT–CDDP inhibits the viability of prostate cancer cells (PC3 and DU145) *in vivo*. However, the effect of released CDDP from SWCNT is not greater than that of bare CDDP, which may be attributed to the loss of CDDP's activity during encapsulation.

The specific destruction of head and neck squamous carcinoma cells (HNSCC) *in vivo* and *in vitro*, directed by the recognition of epidermal growth factor (EGF) by overexpressed EGF receptor (EGFR) on cancer cells, has been demonstrated using a SWCNT-based CDDP delivery system [70,71]. SWCNT–CDDP–EGF treated mice rapidly inhibit tumor growth in comparison to non-targeted SWCNT–CDDP.

In another approach, a Pt(IV) anticancer DDS used soluble SWCNTs as nanovector to transport Pt (IV) prodrug across the cell membrane [71]. Phospholipid (PL)–PEG functionalization of SWCNTs increases the solubility of SWCNTs and extends the functional group away from the nanotube's surface. The Pt (IV) prodrug (c,c,t-[$Pt(NH_3)_2Cl_2(OEt)(O_2CCH_2CH_2CO_2H)$]) forms amide linkages with the PEG-tethered primary amines on the SWCNT surface through heterobifunctional crosslinking using 1-ethyl-3-[dimethylamino)propyl]carbodiimide hydrochloride and *N*-hydroxysuccinimide. The Pt (IV) prodrug internalized by soluble SWCNTs is sixfold more concentrated than unconjugated Pt (IV) prodrug. The lower pH environment within the endosomes promotes the release of Pt(IV) prodrug as *cis*-[$Pt(NH_3)_2Cl_2$], which is the key anticancer drug. Therefore, SWCNTs deliver the Pt (IV) prodrug into cancer cells where they are released as active Pt (II) species.

Similarly, another Pt based antitumor drug, carboplatin (CP), can be incorporated inside CNTs and the effectiveness of drug-filled CNTs on the growth of cancer cells was studied [72]. CP retains its structure inside CNTs and effectively suppresses the growth of bladder cancer cells, whereas CNTs per se do not influence the growth of tumor cells, thus confirming the absence of any intrinsic cytotoxicity.

### 3.1.3. *Other anticancer drugs*

An antitumor DDS, combining biocompatible *f*-SWCNTs, tumor-targeting modules and prodrug modules (taxoid with a cleavable disulfide linker), demonstrated high potency towards specific cancer cell lines [73]. The prodrug is activated to its cytotoxic form inside the tumor cells, upon its internalization and *in situ* drug release. The attachment of biotin and a spacer serves as tumor-recognition modules on the surface of CNT. The specificity and cytotoxicity of the biotin-SWCNT-linker-taxoid conjugate is assessed and compared in L1210 leukemia and human noncancerous cell lines.

In a different study, the colorectal cancer cells can be rapidly heated to 42 °C in 10 s using infrared (IR) radiation based stimulation of oxaliplatin- or mitomycin C-modified CNTs [74]. The photothermal DDS enhances drug localization in cancer cells. The rapid heating is as efficient as the radiative heating for 2 h at 42 °C in the treatment of peritoneal dissemination of colorectal cancer.

MWCNT bound covalently to 10-hydroxycamptothecin (HCPT) using diaminotriethylene glycol as a hydrophilic spacer [75] exhibits better anticancer activity *in vitro* and *in vivo* than the clinical HCPT, and a relatively longer blood circulation apart from high concentration at tumor sites.

CNTs can also incorporate fluorescent agents for biomedical imaging. A *f*-MWCNT-based DDS was developed for the early diagnosis and treatment of cancer [76]. Quantum dot (QD)-conjugated MWCNTs are used for *in vivo* imaging of live mice. Paclitaxel ($112.5 \pm 5.8$ μg per mg C) loaded on CNTs coated with poly(lactic-co-glycolic acid) films exhibits an *in vitro* inhibiting effect on human cancer cells.

N-functionalized pyrrolidine rings can be introduced on the side walls of CNT by 1,3-dipolar cycloaddition [48]. A fluorescent probe and methotrexate (MTX), an antitumor drug, are incorporated around CNT walls by controllable

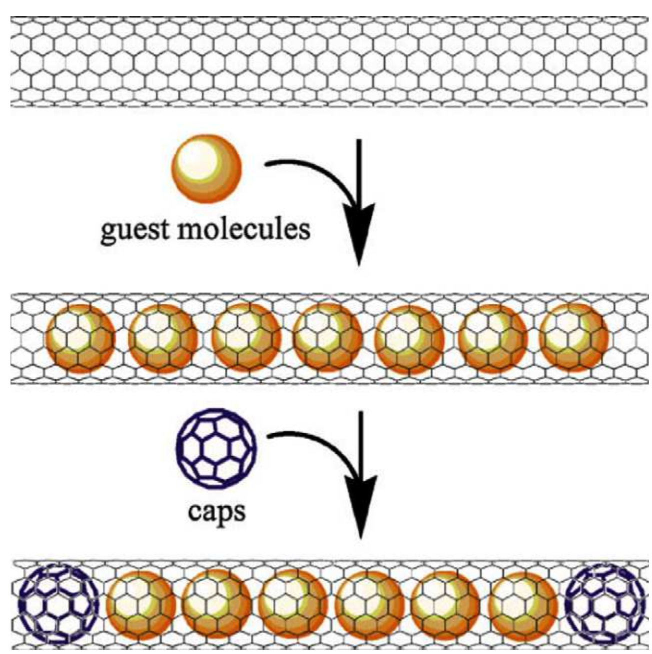


**Fig. 5 – Preparation of "carbon nano-bottles" loaded with antitumor agents and $C_{60}$ using a controlled nano-extraction strategy. $C_{60}$ filled at the extremities of CNTs could act as "cap" to seal the CNTs [79]. Reprinted with permission from Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim.**

Table 2 – Delivery of anticancer drugs.

| Drug delivery system (Drug delivered is in bold) | Dosage (Biological system employed) | Tumor-targeted modules | Trigger | Tumor | Drug-CNT conjugate in comparison to drug | Ref. |
|---|---|---|---|---|---|---|
| Monoclonal antibody-DOX-fluorescein-BSA-SWCNT | N.M.* (WiDr colon cancer cells) | Monoclonal antibody | pH triggered drug release after the interaction of monoclonal antibody with CEA. | Colon cancer | Enable molecular targeting | [61] |
| **DOX**-FA-CHI/ALG-SWCNT | 50 μg $mL^{-1}$ DOX-FA-CHI/ALG-SWCNT (HeLa cells) | FA | FA–FA receptor interaction, pH triggered drug release | Cervical carcinoma | More cytotoxic and selective | [62] |
| Fluorescein-PL-PEG-SWCNT-RGD peptide-**DOX** | 10 μM PL-SWCNT-RGD–DOX (MCF cells) | RGD peptide | RGD peptide recognition moiety-integrin $\alpha_v$-$\beta_3$ receptors, pH triggered drug release | Breast cancer | Less toxic to MCF cells | [39] |
| **DOX**-PL-PEG-SWCNT | 10 mg $kg^{-1}$ DOX–CNT (SCID mice) | N.M.* | pH triggered drug release | Lymphoma | More efficient at treating tumors and less toxic to mice | [63] |
| **DOX**-pluronic F127-MWCNT | 10 μg $mL^{-1}$ DOX:20 μg $mL^{-1}$ CNT (MCF-7 cells) | N.M.* | N.M.* | Breast cancer | More efficient | [64] |
| **DOX**-amphiphilic polymers-CNT | 0.5 mg $mL^{-1}$ DOX-CNT (B16F10 cells) | N.M.* | N.M.* | Melanoma | More efficient | [65] |
| **DOX**/FA-MWCNT@Fe | 32 μg DOX per mg of **FA-MWCNT@Fe** (HeLa cells) | FA and Fe | FA–FA receptor interaction (active) and magnetic force (passive) | N.M.* | Prolonged drug release | [66] |
| **EPI**-c-MWCNT | 131.3–120.8 mg EPI per gram of c-MWCNT | N.M.* | N.M.* | N.M.* | Greater EPI release in acidic medium | [67] |
| **DOX**/PEGylated MWCNT | N.M.* (Hela, HepG2, K562 cells) | N.M.* | N.M.* | Liver cancer and leukemia | Efficient anti-MDR effect | [68] |
| **CDDP**–SWCNT | 100 μg $mL^{-1}$ CDDP-CNT (DU145 and PC3 cells) | CDDP | CDDP-polynucleotide chain interaction | Prostate cancer | Similar effect on PC3 cells but less on DU145 cells | [69] |
| EGF–**CDDP**–SWCNT | 1.3 μM CDDP in EGF-CDDP-SWCNT (Female athymic (nu/nu) nude mice (4–6 weeks old, weighing 18–20 g) | CDDP and EGF | EGF–EGF receptor interaction | Squamous carcinoma | More efficient | [70] |
| {**Pt(IV)**}-PL-PEG-SWCNT | 65 pint(IV) centers per nanotube (average), (NTera-2 cells) | N.M.* | pH triggered drug release | Testicular cancer | Higher toxic to tumor cells | [71] |
| **CP**-MWCNT | 0.5 μg $\mu L^{-1}$ CP-CNT (EJ28 cell line) | N.M.* | N.M.* | Bladder cancer | N.M.* | [72] |
| Biotin-SWCNT-cleavable disulfide linker-(**taxoid**-fluorescein) | 13.9 μM taxoid in 100 μg $mL^{-1}$ conjugate (L1210FR, L1210 and WI38 cell lines) | Biotin | Biotin–biotin receptors mediated endocytosis | Leukemia | More efficient | [73] |
| **Oxaliplatin/MMC**-MWCNT | (300 μM oxaliplatin + 100 μg CNT) per mL medium (RKO and HCT 116 cell lines) | N.M.* | IR radiation stimulated, hyperthermic method | Colorectal cancer | N.M.* | [74] |
| **HCPT**-diaminotriethylene glycol-MWCNTs | 5 mg $kg^{-1}$ HCPT (Hepatic H22 tumor-bearing mice) | N.M.* | pH triggered drug release | Gastric carcinoma | More efficient | [75] |

(continued on next page)

Table 2 – (continued)

| Drug delivery system (Drug delivered is in bold) | Dosage (Biological system employed) | Tumor-targeted modules | Trigger | Tumor | Drug-CNT conjugate in comparison to drug | Ref. |
|---|---|---|---|---|---|---|
| **Paclitaxel**-ultrathin PLGA film-QD-MWCNT | 100 ng $mL^{-1}$ paclitaxel-PLGA-CNT (Nu/nu nude mice; 6–8 weeks old, about 18 g) | N.M.* | N.M.* | Prostate carcinoma | More efficient in tumor treating and low toxic to living mice | [76] |
| $C_{60}$-**HMM**-SWCNT/DWCNT | N.M.* | N.M.* | $CH_2Cl_2$ triggered caps and drugs removal | N.M.* | N.M.* | [79] |
| **MTX** and 1,3-dipolar cycloaddition on MWCNT | 5 μg $mL^{-1}$ conjugate (Jurkat cells) | N.M.* | N.M.* | Jurkat cells | N.M.* | [48] |
| MTX-Gly-Leu-Phe-Gly/6-hydroxyhexanoic ester on 1,3-dipolar cycloaddition ƒ-MWCNT | 10 mM MTX–MWCNT (MCF-7 cells) | N.M.* | N.M.* | Human breast carcinoma | Higher cytotoxicity in MTX–MWCNT using peptide linker | [78] |

N.M.*, not mentioned.

routes, and it is observed that the MTX–CNT complex can rapidly enter Jurkat cells. The same group also demonstrated that ƒ-CNTs, which have undergone similar cycloaddition and oxidation/amidation treatment, are not cytotoxic and preserved the functionality of immune cells [77]. The dependance of anticancer activity of MTX–MWCNT conjugates formed using two different cleavable linkers, i.e. tetrapeptide Gly–Leu–Phe–Gly and 6-hydroxyhexanoic ester, was studied [78]. MTX–MWCNT conjugate, formed by the peptide linker that is selectively cleavable by proteases overexpressed in tumor cells, has higher cytotoxic activity than MTX, ƒ-MWCNT or MTX–MWCNT conjugate formed by the ester linker.

Hexamethylmelamine (HMM), an antitumor agent, can be incorporated inside $C_{60}$ capped SWCNT/double wall carbon nanotubes (DWCNT) [79] (Fig. 5). A "carbon nano-bottle" structure is obtained by sealing CNT opened ends using $C_{60}$ after loading HMM. Therefore, $C_{60}$ can be an important ingredient to seal compounds, which help in the retention of guest molecules inside CNT while protecting them from plausible deactivation. Table 2 provides a summary of CNT-based DDS employed for the delivery of anticancer drugs.

### 3.2. *Delivery of other drugs*

Apart from anticancer drugs, CNT-based DDS have also been employed for the delivery of other drugs (Table 3). Dapsone (dap), an anti-microbial and anti-inflammatory drug, was modified onto ƒ-MWCNTs [80]. There is non-obvious apoptosis of rat peritoneal macrophages when dap-CNTs or oxidized CNTs (o-CNTs), up to 50 μg $mL^{-1}$, are used. Higher levels of both types of CNTs induce apoptosis, which is greater in the case of o-CNTs. However, prolonged incubation of cells (>3 days) in 50 μg $mL^{-1}$ of dap-CNTs triggers apoptosis. Similar levels of individual dapsone and o-CNTs cause oxidative stress, whereas dap-CNTs do not cause any oxidative stress. Therefore, dap-CNTs can be effectively used for treating dap-sensitive intracellular microorganisms and dap-responsive inflammatory diseases.

Dexamethasone (DEX) is a widely used anti-inflammatory and immunosuppressant drug for treating many inflammatory and autoimmune diseases. CHI and SWCNTs can be used as host-carrier films for the electrically stimulated delivery of DEX [81]. An accelerated cellular uptake and a complete drug release are obtained due to electrostatic repulsions of SWCNTs and DEX when −0.8 V vs. Ag/AgCl is applied. The passive release of DEX, i.e. without any stimulation, decreases by the addition of SWCNTs, due to possible attractive interactions between the drug and SWCNTs. The application of a positive potential (+0.15 V vs. Ag/AgCl) to the CHI–CNT–DEX composite decreases the release of DEX.

Ketoprofen, one of the non-steroidal anti-inflammatory drugs with analgesic and antipyretic effects, inhibits the production of prostaglandin in the body. It is commonly prescribed for the treatment of inflammatory conditions due to arthritis or severe toothaches caused by gum inflammation. An electro-sensitive transdermal DDS, composed of a semi-interpenetrating polymer network (polyethylene oxide-pentaerythritol triacrylate) as the matrix and MWCNTs to increase the electrical sensitivity, was demonstrated for (S)-(+)-keto-

**Table 3 – Delivery of other drugs.**

| Drug delivery system (Drug delivered is in bold) | Dosage (Biological system employed) | Drug effect | Drug delivery methods | Drug-CNT conjugate in comparison to drug | Ref. |
|---|---|---|---|---|---|
| **Dopsone**-O-(7-azabenzotriazol-1-yl)-N,N,N′,N′-tetramethyluronium hexafluorophosphate/N,N-diisopropylethylamine-*f*-MWCNT | 50 μg dopsone per mL of *f*-MWCNT (rat peritoneal macrophages) | Anti-microbial and anti-inflammatory | N.M.* | More efficient | [80] |
| Polyethylene oxide-pentaerythritol triacrylate-[(S)-(+)-**ketoprofen**]-MWCNT | N.M.* (Mouse membrane) | Anti-inflammatory | Electrospinning | More efficient | [82] |
| **DEX**–CHI–SWCNT | 0.5 mg per mL CHI (N.M.*) | Anti-inflammatory | Electrical stimulation | More efficient | [81] |
| **AmB**-fluorescein-MWCNT | 40 μg $mL^{-1}$ AmB-CNT (Human Jurkat lymphoma T cells) | Antibiotic | N.M.* | More efficient | [85] |
| **TPGS**–MWCNT | 2.5 μM TPGS (N.M.*) | Vitamin E delivery | N.M.* | N.M.* | [86] |
| **CAR**–MWCNT | 20–60% (wt.%) CAR per drug-CNT complex | Anti-hypertensive | N.M.* | More efficient | [87] |
| **Theophylline**-AL/CNT microsphere | 20% (wt.%) theophylline per drug-CNT complex | N.M.* | N.M.* | More efficient | [88] |
| **Ach**-SWCNT | 20–50 mg $kg^{-1}$ Ach-CNT (Ach: 4–10 mg $kg^{-1}$), (30 Alzheimer's disease mice; 25–30 g; 9 weeks old) | Alzheimer's disease therapeutic agent | pH stimulation | More efficient | [89] |

N.M.*, not mentioned.

profen [82]. The amount of released drug increases with enhanced applied potentials, which can be attributed to higher electrical conductivity of CNTs.

Amphotericin B (AmB), a polyene antifungal drug, is often administrated intravenously for the treatment of systemic fungal infections. However, this drug has serious and potentially lethal side effects to mammalian cells [83]. The toxicity of this drug may be due to its low water solubility that results in the formation of aggregates [84]. The binding of AmB to *f*-CNT can increase its solubility and prevent its aggregation. Also, the drug efficacy will be improved and the antibiotic activity can be modulated. *f*-MWCNTs can be used for the targeted delivery of AmB [85]. MWCNTs are treated with acid to induce carboxylic groups and then functionalized with two orthogonally-protected amino acids. Fluorescein isothiocyanate (FITC) and AmB are conjugated to *f*-MWCNT. AmB preserves its high antifungal activity even after binding to MWCNT and the AmB-CNT complex is transported across the mammalian cells without causing any cytotoxicity.

Tocopheryl polyethylene glycol succinate (TPGS) is a synthetic amphiphile, which is able to deliver α-tocopherol (vitamin E) upon enzymatic cleavage. It is approved by FDA as a nutritional supplement and drug delivery vehicle for vitamin E. TPGS is able to disperse MWCNTs and SWCNTs in aqueous media [86]. Therefore, it is promising for MWCNTs processing due to its ability to effectively disperse MWCNTs at mass ratios (TPGS: MWCNTs) of 1:4 or greater. Its ability to disperse MWCNTs is even more effective than Triton, a commonly used dispersion agent.

Carvedilol (CAR) is a poorly water-soluble drug that is employed for the treatment of hypertension. Various methods were recently attempted for loading CAR in the carboxyl MWCNTs [87], where more CAR is found inside the carboxyl MWCNTs using the solvent method. The solubility of CAR is increased further if it is loaded in *f*-MWCNTs than in MWCNTs, thereby indicating an improvement in its biocompability.

Theophylline was encapsulated in a CNT-filled alginate (AL) microsphere [88]. The drug leakage decreases when AL/CNT microsphere is used in comparison to AL microsphere. The AL/CNT microsphere inherits the pH sensitivity of the AL microsphere and has a more sustainable drug release profile. However, the cytotoxicity of AL/CNT microsphere is similar to that of AL microsphere.

Acetylcholine (Ach) is an important neurotransmitter in the peripheral and central nervous system in many organisms including humans. The delivery of Ach into the brain may be useful for the treatment of Alzheimer's disease. Lysosomes and mitochondria are identified as the pharmacological and toxicological target organelles, respectively for SWCNTs [89]. Therefore, SWCNTs are utilized to release Ach into the brain for treating the experimentally induced Alzheimer's disease with a moderate safety range. This is done by precisely controlling the doses, ensuring that SWCNTs preferentially enter lysosomes but not mitochondria.

## 4. Delivery of biomolecules

### 4.1. DNA and RNA

DNA can be attached to the amino groups of *f*-MWCNT [47]. The linkage of DNA to *f*-MWCNT is utilized for improving nanotubes' dispersibility in aqueous media as well as for efficient gene transfection without the use of viral genes.

Polyethylenimine (PEI) can be grafted onto MWCNT to form (PEI-g-MWCNT) complex, which is used for the immobilization and release of DNA [40]. The grafted PEI has high contents of primary, secondary and tertiary amines for immobilizing DNA onto MWCNT. PEI-g-MWCNT exhibit a good transporting efficiency for the delivery of DNA. However, pristine or amine-*f*-MWCNTs have a little effect on DNA migration.

MWCNTs functionalized with cationic polyelectrolyte were used for the intracellular delivery of antisense oligode-

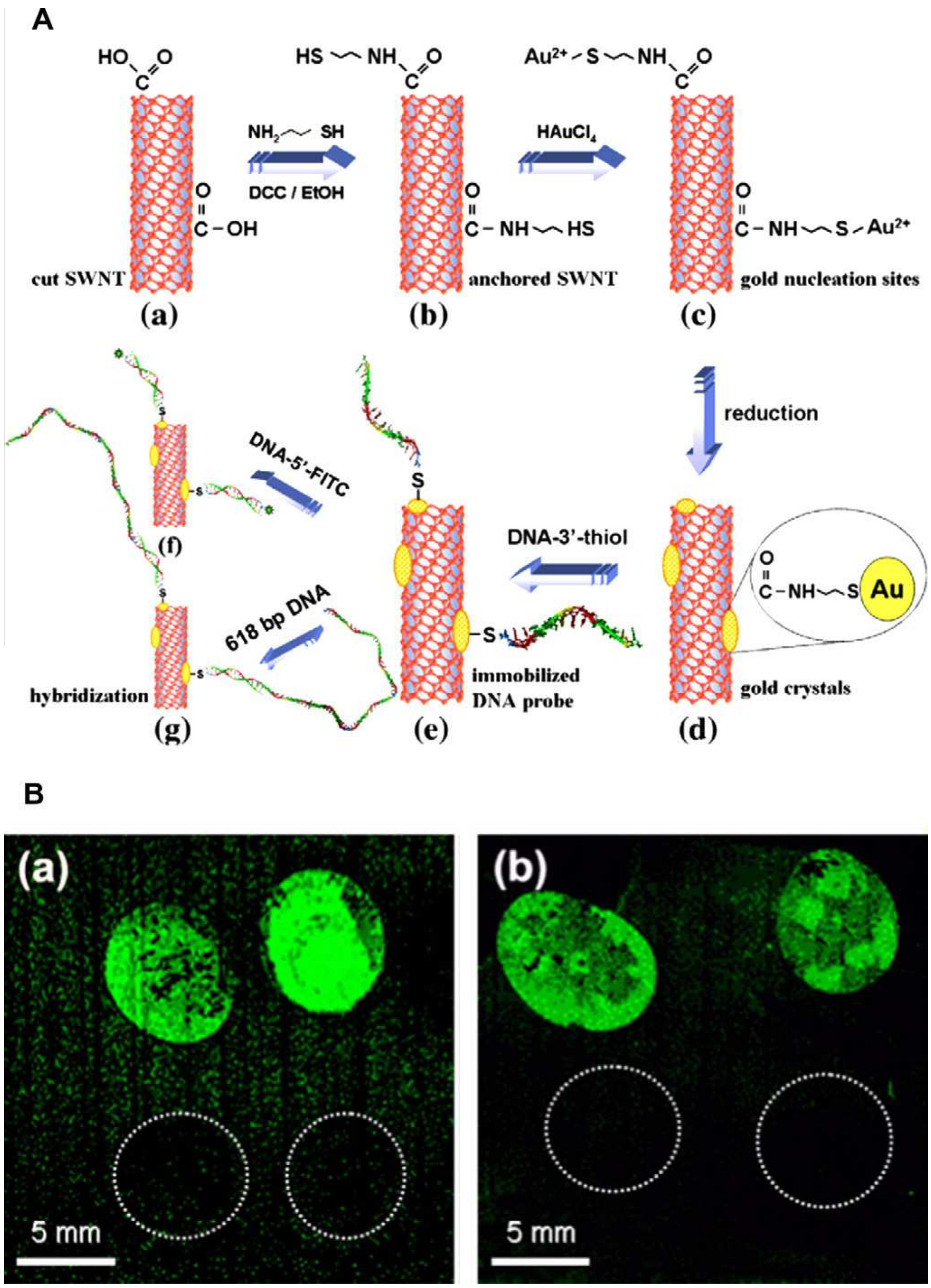


**Fig. 6 – (A) Overall scheme for fabrication of Au-SWCNTs, and the immobilization and hybridization of DNA. (B) Scanned fluorescent images of hybridization of (a) the FITC-labeled complementary oligonucleotide with probe DNA (above); the non-complementary oligonucleotide (below). (b) Hybridization of the FITC-labeled complementary oligonucleotide to probe DNA (above) and Au-SWCNT without probe DNA (below) [93]. Reprinted with permission from Elsevier.**

oxynucleotides (ASODN) [45]. Mercaptoacetic acid-capped CdTe QD are used as fluorescent probes to image the transport of ASODN for determining their efficiency of release. PEI-MWCNTs have high intracellular transport efficiency, strong cell nucleus localization and high ASODN delivery efficiency. Moreover, ASODN bound to PEI-MWCNT show effective anticancer activity.

The "CNT spearing" technique was developed for the effective molecular delivery based on the transportation of nickel (Ni) embedded MWCNTs into the cell membranes [90]. The transportation is driven by an external magnetic field. DNA plasmids, including a green fluorescent protein (GFP) sequence, are bound to MWCNT followed by the spearing of DNA–MWCNT into the targeted cells. The use of MWCNT spearing technique results in higher transduction efficiency and higher viability after transduction in Bal17 B-lymphoma, *ex vivo* B cells and primary neurons.

The release of GFP gene to human umbilical vein endothelial cells (HUVEC) and A375 cells (a human melanoma cell line) was studied [91]. $NH_2$ group *f*-MWCNTs effectively deliver the pEGFPN1 plasmid into the cells. However, carboxyl-, hydroxyl-, or alkyl- *f*-MWCNTs are not capable of releasing the pEGFPN1.

DNA binds to SWCNTs and can be effectively released into HeLa cells by the cleavage of a disulfide bond between *f*-SWCNT and DNA in the cytosol followed by its nuclear translocation [92]. The transportation of DNA by SWCNTs inside the two cell lines, i.e. adherent HeLa and non-adherent HL60 cells, is also studied. The successful uptake of the DNA-SWCNT conjugate by HeLa and HL60 cells, suggests internalization by energy-dependent endocytosis.

SWCNTs dotted with Au nanocrystals (Au-SWCNT) were developed and employed for the delivery of DNA (Fig. 6) [93]. DNA probes functionalized with a thiol group at the 3′ end are conjugated to Au-SWCNT. The hybridizion of complementary oligonucleotides is detected and verified by fluorescence-based measurement. Atomic force microscopy (AFM) images confirmed specific DNA hybridization.

The adsorption and delivery of single-stranded DNA wrapped SWCNTs (ssDNA-w-SWCNT) on insulating self-assembled monolayer (SAM) was also evaluated [94]. The electron transfer between Au and electro-active species blocked by SAM is recovered by employing SWCNT or ssDNA-w-SWCNT. The delivery of ssDNA-w-SWCNT is also controlled by applying a positive or negative potential to the ssDNA-w-SWCNT/Au electrode.

SWCNT have been advocated as carriers for the intracellular delivery of ssDNA probe [95]. This strategy can avoid nuclease digestion or protein interaction, thus improving the efficiency of transfection. The binding of DNA probes to SWCNTs protect them from enzymatic cleavage and disturbance from nucleic acid binding proteins. SWCNT bound DNA probes, which bind to a specific target mRNA, has improved self-delivery and intercellular biostability in comparison to free DNA probes.

Cationic glycopolymers-*f*-SWCNTs were developed as efficient gene delivery vehicles for *in vitro* gene transfer [96]. The biocompatibility and transfection efficiencies of copolymer-functionalized SWCNTs are comparable with lipofectamine 2000, a commercially available gene delivery agent.

Cationic SWCNTs are bound to the synthetic oligodeoxynucleotides with CpG motifs (ODN CpG) [50]. *f*-SWCNTs enhance the immunostimulation of ODN CpG *in vitro*, which can be attributed to the decrease of repulsions between negatively charged ODN CpG membrane and positively charged SWCNT.

Oxidized ultrashort SWCNTs are used as scaffolds to improve the intracellular delivery of ODN decoys inhibiting nuclear factor-κB (NF-κB), a transcription factor regulating many genes involved in immunity. The effective binding of amino-modified ODNs to COOH groups introduced on SWCNT significantly reduces the NF-κB-dependent gene expression in cells receiving nanomolar concentrations of SWCNT-NF-κB decoys than in those receiving SWCNT or SWCNT functionalized with nonspecific ODNs [97].ODN were bound covalently to the external sidewalls of SWCNT [98] and their highly specific and reversible hybridization to the complementary target DNA strand were demonstrated.

Multi-*f*-SWCNTs containing a FA moiety were employed for the near-infrared (NIR) stimulated destruction of cancer cells [35]. ODN transport into cells by binding to CNT and translocate inside the cell nucleus when triggered by NIR laser pulses. The increase of NIR radiation provokes cell death due to the excessive local heating from CNTs. The FA moiety on CNT facilitates the selective death of cancer cells as it interacts with the folate receptor present on the surface of tumor cells.

A supramolecular hybrid was fabricated by the functionalization of SWCNTs with β-cyclodextrin-tethered ruthenium via a spacer molecule containing adamantane and a pyrene moiety [99]. The introduction of the supramolecular hybrid enables the control of spatial condensation of negative DNA upon the SWCNT skeleton by loading cationic ruthenium on the surface. The ruthenium complex can function as a fluorescent probe to detect the cellular uptake of DNA.

A water-soluble SWCNT–DNA covalent complex was prepared by carbodiimide-assisted amidation [100]. SWCNT–DNA complexes are capable of hybridizing selectively with complementary DNA sequences without any nonspecific interactions with non-complementary DNA strands.

The physicochemical interactions between ammonium-*f*-SWCNT/MWCNT (SWCNT-$NH_3^+$, MWCNT-$NH_3^+$), lysine-*f*-SWCNT (SWCNT-Lys-$NH_3^+$), and plasmid DNA were investigated [101]. All *f*-CNTs condense DNA to varying degrees and upregulate marker gene expression over free DNA in a human cell line.

The positively charged ammonium *f*-SWCNTs/MWCNTs were used for conjugating plasmid DNA [102]. DNA–CNT complexes bind to the cells and penetrate them by an endosome-independent mechanism. These complexes also facilitate a higher DNA uptake and gene expression *in vitro* in comparison to DNA without CNT.

The small interfering RNA (siRNA) delivery by two types of *f*-MWCNTs i.e. MWCNT-PEI and MWCNT-pyridinium [103] was recently compared. Both types of *f*-MWCNTs show 10–30% silencing activity and 10–60% cytotoxicity. However, MWCNT-PEI and MWCNT-pyridinium do not show any superior performance, in terms of reduced toxicity and increased silencing activity, in comparison to PEI or other standard transfection systems.

SWCNTs could also be functionalized by covalent binding with hexamethylenediamine (HMDA) and poly(diallyldimethylammonium) chloride (PDDA), which then bind to negatively charged small interfering RNA (siRNA) by electrostatic attractions [104]. PDDA–HMDA–SWCNT functionalized with extracellular signal-regulated kinase (ERK) siRNA penetrates the cell membrane and inhibits the expression of ERK target proteins by about 75% in primary cardiomyocytes.

SWCNTs were also used for the release of siRNA to provide effective RNA interference (RNAi) of CXCR4 and CD4 receptors on human T cells and peripheral blood mononuclear cells (PBMC) [44]. The delivery and RNAi capability of SWCNTs exceeds that of liposomes (Lipo1–4, existing nonviral transfection agents). SWCNTs with relatively long length (ca. 200 nm) promote binding with the hydrophobic domain of cell membranes by hydrophobic interactions.

Short SWCNTs were functionalized with PL-$PEG_{2000}$, followed by the incorporation of disulfide bonds and then their conjugation to siRNA [46]. The siRNA is released from SWCNT by the enzymatic disulfide cleavage inside HeLa cells. The silencing efficiency of siRNA-CNT conjugates is twofold better than that of lipofectamine.

SWCNT-CONH-$(CH_2)_6$-$NH_3^+Cl^-$ improves the binding of siRNAs targeting murine TERT (mTERT) expression to fabricate mTERT siRNA–SWCNTs [105]. These siRNA–SWCNTs are rapidly transported into three murine tumor cell lines, suppress mTERT expression, and arrest cell growth. The injection of siRNA–SWCNTs into lung cancer cells suppresses the tumor growth. The human TERT siRNA–SWCNT complex also suppresses the human HeLa cell growth both *in vitro* and in tumor cells in mice.

### 4.2. Proteins

BSA can be bound covalently to SWCNTs/MWCNTs by diimide-activated amidation to form CNT–BSA conjugates with high water solubility [106]. About 90% of BSA molecules retain their activity, as determined by the total protein micro-determination assay.

The internalization of CNT-protein conjugates into mammalian cells was studied by modifying oxidized SWCNT (containing carboxylated groups) with EDC and biotin-LC-PEO-amine, and incubating in fluoresceinated protein streptavidin (SA) to prepare a SWCNT biotin-SA complex [107]. EDC–SWCNT are able to transport into HL60, CHO and 3T3. The fluoresceinated protein SA enters the cells after binding to SWCNT-biotin transporter, but cannot enter the cells by itself under the same experimental conditions. The SA-SWCNT-biotin complex exhibits dose-dependent cytotoxicity after internalization.

The noncovalent and nonspecific binding of various types of proteins (molecular weight $\leqslant$80 kD) to the sidewalls of SWCNT was reported [41]. The protein transport and its uptake through CNT carriers are generic for varied adherent and nonadherent cell lines. The internalization occurs by energy-dependent endocytosis. Apoptosis is provoked by cyt-c, which is transported inside the cells with SWCNT and then released from the endosomes. *In vitro* biological functionality and the activity of proteins delivered by SWCNT are also demonstrated. The same group also reported the successful cellular uptake of BSA and SA-SWCNTs by HeLa and HL60 cells [92]. The noncovalent conjugation between proteins and SWCNTs is sufficiently strong for their transport as carrier-cargo complexes into the cells. The cellular internalization mechanism as well as the pathway for protein-SWCNT complexes is also studied.

A non-covalent method was developed to incorporate *f*-SWCNT with ferritin, SA, and biotinyl-3,6-dioxaoctanediamine [108]. 1-Pyrenebutanoic acid and succinimidyl ester are used to modify CNT with pyrenyl and succinimidyl groups, respectively. The pyrenyl groups bind to CNT by strong $\pi$–$\pi$ interactions, whereas the succinimidyl ester groups act as anchors for the binding of proteins.

An interesting investigation of the biochemical pathways involved in the use of CNTs, reveals that CNTs activate human complement via classical and alternative pathways [109]. The complement activation by CNTs corresponds to the reported adjuvant effects and may enhance the damaging consequences of excessive activation (e.g. inflammation, granuloma formation, etc.). Fibrinogen and apolipoproteins (AI, AIV and CIII) in serum and plasma bind to CNTs in greater quantity.

Two different procedures for the preparation of the peptide-CNT conjugate were developed based on fragment condensation and selective chemical ligation [53]. Peptides are linked to CNT by a stable covalent bond. The bound peptide from the foot-and-mouth disease virus (FMDV) preserves its structural integrity and can be recognized by antibodies. Moreover, this peptide-CNT complex is immunogenic and elicits specific antibody response. In a different study, a neutralizing B cell epitope from the FMDV was covalently linked to mono- and bis-derivatized CNT [51]. The immunological detection of these complexes shows that the epitope is recognized by antibodies after its conjugation with CNT. In fact, mono-derivatized CNT complex can provoke high levels of virus-defending antibodies. These experimental results are highly valuable as they highlight for the first time the application of CNTs in presenting biologically important epitopes both *in vitro* and *in vivo*.

The translocation of peptides across the cell membranes with the help of CNTs was also reported [52]. The water-soluble SWCNTs functionalized with a fluorescent probe translocate across the cell membranes. The peptide responsible for the activity of G protein, an important protein for signal transduction, can penetrate into the cell when it is covalently bound to SWCNT.

GRGDSP, a fibronectin-derived peptide, and IKVAV, a laminin-derived peptide can be conjugated to soluble *f*-MWCNTs [110]. The *f*-MWCNTs exhibit biocompatibility with different cell types, and do not seem to change the neuronal morphology, viability, and basic functions.

The *in vitro* ingestion and loading ability of MWCNTs in microglia, and the differences in the internalization of CNTs by BV2 microglia and GL261 glioma cells was also studied [111]. CNTs do not lead to *in vitro* cell proliferation or cytokine changes. DNA or siRNA carried by these CNTs is internalized at higher levels in phagocytic cells than in tumor cells.

**Table 4 – Delivery of biomolecules.**

| Delivery system (Biomolecules in bold) | Biological system employed | Results | Ref. |
|---|---|---|---|
| **BSA/DNA**-amino-MWCNT | HeLa and HL60 cells | BSA and DNA were covalently bound to amino-MWCNT | [47] |
| **DNA**-PEI-MWCNT | 293cells, COS7 and HepG2 cells | PEI served as anchor points for DNA immobilization; PEI-g-MWCNT exhibited good transfection efficiency for the delivery of DNA | [40] |
| **ASODNs**-PEI-MWCNT | HeLa cells | ASODN interacted with positively charged amine groups on PEI-MWCNT | [45] |
| **Plasmid DNA**-carboxylic *f*-MWCNT with embedded Ni | Bal17 B-lymphoma, *ex vivo* B cells and primary neurons | DNA-MWCNT entered in Bal17 B-lymphoma, *ex vivo* B cells and primary neurons driven by magnetic field and remained high viable even after transduction | [90] |
| **GFP gene**-Amino/carboxyl/hydroxyl/alkyl-MWCNT | Human umbilical vein endothelial cells (HUVEC) | Only amino group functionalized MWCNT effectively delivered the pEGFPN1 plasmid into cells | [91] |
| **BSA/SA/DNA**-carboxyl-SWCNT | | Protein-SWCNT entered into the living cells as carrier-cargo complexes; uptake mechanism was energy-dependent endocytosis; pathway was mainly by clathrin-coated pits | [92] |
| **ssDNA**-SWCNT dotted with Au nanocrystals (Au-SWCNT) | N.M.* | target DNA hybridization to ssDNA probes, which were immobilized on Au-SWCNT | [93] |
| **ssDNA**-pristine SWCNT | N.M.* | ssDNA bound to SWCNT got released by desorption potential | [94] |
| **ssDNA**-carboxyl-SWCNT | MDAMB-231 breast carcinoma cells | CNT-modified DNA probe binds to a specific target mRNA inside living cells with increased self-delivery and intracellular biostability | [95] |
| **DNA**-diblock copolymers P(APMA$_{38}$-*b*-GAPMA$_{20}$)-SWCNT | Hela cells | The biocompatibility and transfection ability of SWCNTs was comparable with lipofectamine 2000 | [96] |
| **ODN CpG** and 1,3-dipolar cycloaddition on SWCNT | N.M.* | *f*-SWCNT enhanced immunostimulatory properties of ODN CpG; Concentration of IL-6 (stimulated by ODN CpG combined with *f*-SWCNT) in splenocyte cultures decreased more | [50] |
| **NF-κB decoy-SWCNT** | HeLa cells | Covalent binding of NF-κB decoy on SWCNT greatly reduced the NF-κB dependent gene expression | [97] |
| **ODN**-SWCNT with maleimide terminal group | N.M.* | Hybridization of complementary DNA was highly specific and reversible | [98] |
| **ODN**-FA-PEG-PL-SWCNT | HeLa cells | CNT complex translocated inside cell nucleus triggered by NIR laser pulses; increase of NIR radiation provoked tumor cell death | [35] |
| **DNA**-[Ru-(phen)$_2$(β-CD-hophen)]Cl$_2$ ((β-CD-CR), adamantane derivatives (Py-Ad)-SWCNT | Yeast cells | Spatially controllable DNA condensation along SWCNT skeleton was obtained; ruthenium complex acted as a fluorescent probe to detect the cellular uptake of DNA | [99] |
| **DNA**-carbodiimide group *f*-SWCNT | N.M.* | Complementary DNA sequence selectively hybridized to DNA bound on SWCNT | [100] |
| **Plasmid DNA** and 1,3-dipolar cycloaddition on SWCNT/MWCNT | A549 cells | SWCNT-$NH_3^+$, MWCNT-$NH_3^+$, SWCNT-Lys-$NH_3^+$ condensed DNA to varying degrees; they also exhibited upregulation of marker gene expression over free DNA | [101] |
| **Plasmid DNA** and 1,3-dipolar cycloaddition on SWCNT/MWCNT | HeLa cells | *f*-SWCNT complexed with plasmid DNA facilitated higher DNA uptake and gene expression *in vitro* | [102] |
| **siRNA**-PEI/pyridinium-*f*-MWCNT | Human lung cancer cell line H1299 | Both types of *f*-MWCNTs showed 10–30% silencing activity and 10–60% cytotoxicity | [103] |
| **siRNA**-PDDA-HMDA-SWCNT | Isolated rat heart cells | PDDA-HMDA-SWCNT bound negatively charged siRNA by electrostatic interactions | [104] |
| **siRNA**-PL-PEG-SWCNT | Human T cells and primary cells | CNT were capable of siRNA delivery to human T cells and PBMCs, and caused RNAi of CXCR4 and CD4 receptors | [44] |
| **siRNA/DNA**-PL-PEG-SWCNT | HeLa cells | Amine or maleimide terminal of PL-PEG-SWCNT could bind to various biomolecules | [46] |

(*continued on next page*)

Table 4 – (*continued*)

| Delivery system (Biomolecules in bold) | Biological system employed | Results | Ref. |
|---|---|---|---|
| **TERT siRNA**-SWCNT–CONH–$(CH_2)_6$-$NH_3^+Cl^-$ | HeLa cells | TERT siRNA specifically targeted TERT expression and led to growth arrest of tumor cells | [105] |
| **Ferritin/SA/biotinyl-3,6-dioxaoctanediamine**-1-Pyrenebutanoic acid, succinimidyl ester-SWCNT | N.M.* | Pyrenyl groups bound to CNT through strong $\pi$–$\pi$ interaction, while succinimidyl ester groups worked as anchors for combining proteins | [108] |
| **BSA**-SWCNT-$CONH_2$ **BSA**-MWCNT-$CONH_2$ | N.M.* | 90% BSA retained activity after the formation of BSA-CNT conjugates | [106] |
| **BSA/SA/Protein A/cytochrome *c* (cyt-*c*)**-carboxyl-SWCNT | HL60, Jurkat, HeLa and NIH-3T3 cells | High level of cellular uptake of proteins (molecular weight <80 KDa); cyt-*c* SWCNT conjugate led to higher level of apoptosis in the presence of chloroquine | [41] |
| **SA**-Biotin-SWCNT | HL60 and Jurkat cells | SA entered cells after binding to SWCNT-biotin transporter | [107] |
| **Protein C1q/serum/plasma proteins**-pistine SWCNT | Red blood cells | CNT activated human complement through both classical and alternative pathways; C1q bound directly to CNT; fibrinogen and apolipoproteins (AI, AIV and CIII) bound selectively to DWCNT | [109] |
| **GRGDSP peptide sequence/IKVAV peptide sequence** and 1,3-dipolar cycloaddition on MWCNT | Jurkat cells, primary splenocytes and neurons | MWCNT exhibited biocompatibility with different cell types; they did not seem to change the neuronal morphology, viability, and basic functions | [110] |
| **KGYYG sequence/GSGVRGDFGSLAPRVARQL sequence** and 1,3-dipolar cycloaddition on SWCNT | N.M.* | Bound peptides were recognized by monoclonal and polyclonal antibodies; peptide-SWCNT caused immune response | [53] |
| **K(FITC)QRMHLRQYELLC sequence** and 1,3-dipolar cycloaddition on SWCNT | 3T3 and 3T6 cells | CNT conjugate crossed the cell membrane; FITC-CNTs accumulated mainly in cytoplasm; Peptide-CNT accumulated in nucleus | [52] |
| **BV2 microglia/GL261 glioma**-pluronic F108-MWCNT | BV2 microglia and GL261 glioma cells | CNT did not lead to proliferative or cytokine changes *in vitro*; they carried DNA and siRNA, and were internalized at higher levels in phagocytic cells than in tumor cells | [111] |
| **Protective B cell epitope** and 1,3-dipolar cycloaddition on SWCNT | BHK 21 cells | B cell epitope was recognized by specific antibodies after being conjugated to SWCNT; mono-peptide-SWCNT led to higher virus neutralizing antibody titers than bis-peptide-SWCNT | [51] |
| **Anti-HER2 IgY antibody**-SWCNT-$CONH_2$ | SK-BR-3 and MCF-7 cells | CNT-antibody complex could detect and selectively kill SK-BR-3 (cancer cells expressing HER2) *in vitro* in the presence of MCF-7 (non-HER2 expressing) cells | [34] |
| **EPO**-PEG-8 caprylic/capric glycerides-CNT | Male Wistar rats | Shorter CNT released twice the amount of EPO than longed CNT in rat serum | [113] |
| **GnRH**-carboxylic-MWCNT | DU 145 cells | GnRH–MWCNT killed Hela cells after internalization by GnRH receptor-positive cells | [114] |

N.M.*, not mentioned.

Anti-HER2 chicken IgY was covalently bound to carboxyl-SWCNT for *in vitro* detection and selective killing of SK-BR-3 (cancer cells expressing HER2) in the presence of MCF-7 (non-HER2 expressing) cells [34]. The detection concept is based on the strong resonance at Raman scattering of SWCNTs [112], while the therapeutic effect is based on the NIR absorbance for the selective photothermal excision of cancer cells [35].

The effect of CNT's fiber length on the absorption of erythropoietin (EPO) was also investigated [113]. PEG-8 caprylic/capric glycerides are employed to improve the absorption of EPO on CNTs. Casein (used as protease inhibitor) and sodium starch glycolate (used as disintegrating agent) are also mixed together to fabricate a solid product. This product is delivered orally to rat and the serum EPO levels are determined. EPO level reaches the maximum value of $69.0 \pm 3.9$ mIU/ml within $3.5 \pm 0.1$ h. However, the use of shorter CNTs as carrier releases twice the amounts of EPO in comparison to that of longer CNTs.

The gonadotropin (GnRH) functionalized carboxylic MWCNTs tended to kill HeLa cells after they were internalized by the GnRH receptor-positive cells [114]. Table 4 provides the summary of CNT-based DDS for the delivery of biomolecules.

## 5. Cytotoxicity of CNTs

The cytotoxicity of CNTs needs to be extensively investigated *in vitro* and *in vivo* if they are employed as drug carriers. There are numbers of research reports focussed exclusively on this issue, but the reported cytotoxicity findings of CNTs are incompatible with each other. These conflicting reports may be attributed to variability in the doses, properties, purification and functionalization of CNTs employed for various cytotoxicity studies. Different types of cell populations and assay methods may also lead to paradoxical findings. Various kinds of cell-viable indicator dyes, such as commassie blue, alamar blue, neutral red, MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide), and WST-1 (a watersoluble tetrazolium salt) [115], are used in the cytotoxicity studies as they can bind to CNTs and result in observable changes in the associated absorption/fluorescent emission, which correspond to the cytotoxic effect of CNTs. These can also lead to variability in the CNT cytotoxicity results.

### 5.1. *Factors affecting the cytotoxicity of CNTs*

The *in vitro* and *in vivo* cytotoxicity studies of CNTs mainly concentrate on the effect of metal catalyst impurities, length and type of CNTs, and different chemistries used for the surface functionalization and dispersion of CNTs. The integrated effect from various factors is also considered. Metal catalysts are the main source of cytotoxicity in CNTs [116–119]. For instance, iron, a most common catalyst for growing CNTs, may boost the free radical reactions in the living cells [120]. There have been contradictory findings, where some reports state that purified CNTs are not cytotoxic, but others claim that refined CNTs may be more toxic [121,122]. The length of CNTs also affects its cytotoxicity. Sato et al. [123] reported that similar slight cytotoxicity is found *in vitro* with MWCNTs of 220 or 825 nm length, while Becker et al. [124] proved that CNTs shorter than $(189 \pm 17)$ nm have greater cytotoxicity. Different types of CNTs, i.e. SWCNTs and MWCNTs, may have different nanotoxicological effects due to their variable surface area [125]. SWCNTs have greater surface area, but they are more prone to aggregate into bundles due to stronger van der Waals forces, thereby causing reduced surface area. The aggregation of CNTs is known to be harmful to the living cells, organs and tissues [126]. Surface area of MWCNTs is slightly lower but there are many active defect sites along their sidewalls that may help to avoid their aggregation. Till date, MWCNTs seem to be less toxic than SWCNTs. However, the actual cytotoxicity comparison of SWCNTs and MWCNTs is difficult as it is not clear whether nanotoxicity should be related to the same mass concentration or the same total surface area of CNTs [115]. Furthermore, CNTs are required to be hydrophilic as drug carriers. Therefore, the surface chemistry plays an important role to improve the biocompatibility of CNTs. A few publications have demonstrated significant reduction in the cytotoxicity of CNTs due to high degree of functionalization on the CNT sidewalls [82,127,128]. *f*-SWCNTs are much less toxic than surfactant-stabilized SWCNTs [77]. In a typical experiment, immunoregulatory cells (e.g. macrophages, B and T lymphocytes) were incubated in two types of amino group *f*-SWCNTs, one being highly soluble and another forming stable suspension in aqueous solution. The activities of the immunoregulatory cells are not influenced by the highly soluble CNTs, whereas proinflammatory cytokines are secreted by macrophages in the CNT suspension. The chemistry used for CNT dispersion also influences their toxicity. Two different dispersion agents, dimethylsulfoxide (DMSO) and 1% Pluronic F127 (anionic surfactant), were used to disperse the 6-aminohexanoic acid derivatized SWCNT (AHA-SWCNT) [129]. One percent Pluronic F127 disperses the aggregation of AHA-SWCNTs more efficiently than DMSO and thus reduces their cytotoxicity. Apart from the factors mentioned above, CNT dose and types of cells and methods employed for the cytotoxic assay also influence the results. Therefore, it is absolutely essential to consider the integrated interactions between all possible factors when a reliable protocol is designed for the *in vitro* or *in vivo* cytotoxic assay.

### 5.2. *Cytotoxicity mechanisms of CNTs*

Several cytotoxicity mechanisms have been proposed with some claiming the cytotoxicity of CNTs due to the disruption of intracellular metabolic pathways, and others stating that CNTs causes oxidative stress and membrane damage. The most developed pattern for determining the effect of CNTs on the mammalian cells is the generation of reactive oxygen species (ROS) due to oxidative stress [130].

SWCNTs may cause secretion of small proteins, accumulation of cells, cell apoptosis and other cell behaviours in the human embryonic kidney cells [117]. MWCNTs may arrest cell-cycle; increase apoptosis/necrosis; perturb cellular pathways; activate the genes involved in the cellular transport, metabolism and cell-cycle regulation; and, induce stress response [118,119]. CNTs can mechanically block the large airways in rat lungs [131] and induce dose-dependent interstitial granulomas and pulmonary injuries in mice [132]. Significantly increased cytotoxicity and inflammatory markers in animal lungs after pharyngeal aspiration of CNTs have also been reported [131]. Diameter- and length-dependent cytotoxic effect of MWCNTs has also been implied in another mice model assay [133]. SWCNTs can form fiber-like structures in mice body and induce granuloma formation once the fiber length increases to 10 μm, regardless of dose or length of the tubes [134]. Individual SWCNTs shorter than 300 nm will not prevent themselves from excretion through kidneys or bile ducts by the reticuloendothelial system, whereas small accumulation of SWCNTs can stay inside cells for 5 months although they do not provoke granuloma formation.

## 6. Conclusions

CNTs have been increasingly attempted for the delivery of drugs and biomolecules in the past decade. Significant advances have been made in the delivery of anticancer and anti-inflammatory drugs, and biomolecules i.e. DNA, RNA and proteins. Drugs and biomolecules can be stored inside CNTs, which can then be bound to targeting molecules such as antibodies or contrast agents. The toxicity of pristine CNTs is still a major concern based on the highly conflicting results obtained by various researchers. Pristine CNTs are highly toxic and insoluble in physiological media. There is a dire need to establish international guidelines for determining the toxicity of nanomaterials including CNTs, which need to be strictly adhered to in all circumstances. However, functionalized CNTs have been considered biocompatible and safe for drug and biomolecular delivery applications as they are soluble in physiological media and nontoxic. They have shown no accumulation in the tissues; conversely, once functionalized, they can be readily excreted through the renal route. The toxicity of CNTs is mainly attributable to impurities, length of CNTs, surface chemistry, dispersion and tendency to aggregate, and interaction between various factors [115].

Overall, the use of CNTs for delivery of drugs and biomolecules is a significant development in the field of therapeutic nanomedicine. The technology development is going on at a very fast pace in this area but still it is too far from becoming a clinical and commercial reality based on the numerous challenges involved. However, CNT-based delivery systems are undoubtedly very promising in terms of their numerous advantages over the existing technologies.

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