

Expert Opinion

1. Introduction
2. Drug delivery and targeting
3. Mechanisms of cell uptake
4. Pharmacokinetic studies
5. Conclusions
6. Expert opinion

The alluring potential of functionalized carbon nanotubes in drug discovery

Cécilia Ménard-Moyon, Enrica Venturelli, Chiara Fabbro, Cristian Samorì, Tatiana Da Ros, Kostas Kostarelos, Maurizio Prato & Alberto Bianco[†]
[†]CNRS, Institut de Biologie Moléculaire et Cellulaire, UPR 9021 Immunologie et Chimie Thérapeutiques, 67000 Strasbourg, France

Importance of the field: The possibility of carbon nanotube integration into living systems for therapeutic and diagnostic purposes has opened the way to explore their applications in drug delivery and discovery. A wide variety of chemical approaches has been developed to functionalize carbon nanotubes with therapeutic molecules towards different biomedical uses.

Areas covered in this review: This review covers the recent advances in the development of functionalized carbon nanotubes to offer improvements for different diseases, in particular for cancer therapy.

What the reader will gain: Functionalized carbon nanotubes are able to transport therapeutic agents. Targeted methodologies using carbon nanotube-based conjugates have been investigated to improve the efficacy of some drugs. The capacity of such nanomaterials to seamlessly translocate into cells with alternative various mechanisms and their pharmacokinetic properties is also discussed.

Take home message: Although at its infancy, functionalized carbon nanotubes are very promising as a new nanomedicine platform in the field of drug discovery and delivery. They have the capacity to cross biological barriers and can be eliminated via renal and/or fecal excretion. They can transport small drug molecules while maintaining – and in some cases improving – their therapeutic efficacy.

Keywords: biodistribution, carbon nanotubes, cell uptake, drug delivery, drug discovery, functionalization

Expert Opin. Drug Discov. (2010) 5(7):691-707

1. Introduction

Carbon nanotubes (CNTs) belong to the family of carbon allotropes. Discovered in the 1950s/1960s [1] and described at atomic resolution in 1991 by Iijima [2], they are constituted of cylinders of graphene sheets opened or closed at the extremities. CNTs can be either composed by a single plane of graphene (single-walled carbon nanotubes, SWCNTs) or by multiple concentric layers (multi-walled carbon nanotubes, MWCNTs). They have diameters in the nanometer range, while their lengths can reach several micrometers (Figure 1).

This new type of nanomaterials is currently developed for a wide variety of uses in materials science [3] and biomedicine [4]. In particular, CNTs can be considered as novel and innovative tools in the development of alternative methodologies for the delivery of therapeutic molecules [5]. On the other hand, nanotube-based conjugates can be considered as novel nanomedicine tools developed in the field of drug discovery. Although CNT technology is considered still young, some advantages are clearly emerging (Figure 2) [6]. CNTs possess a high surface area with a high aspect ratio, ultra-light weight, high mechanical strength, high electrical conductivity, high

informa
healthcare

Article highlights.

- Many studies have shown the potential of functionalized carbon nanotubes (CNTs) as drug delivery systems to maintain and occasionally improve the biological activity of therapeutic molecules.
- Two main routes of cell uptake have been identified: i) direct cytoplasmic translocation ('nanoneedle' mechanism) and ii) phagocytosis/endocytosis processes. Cell internalization depends strongly on the type of CNTs and the functional groups on their surface.
- Functionalized CNTs are generally eliminated into the urine or feces.
- CNTs can be suitable nanovectors for drug delivery applications as they display negligible adverse effects.
- The attractive properties of CNTs can offer new opportunities for drug discovery and delivery over other delivery systems. Nevertheless, more studies are necessary to establish their advantages and limitations.

This box summarizes key points contained in the article.

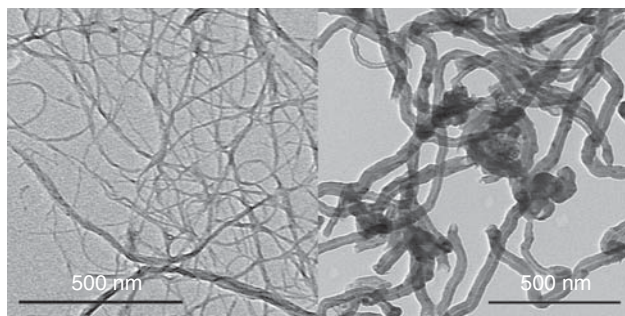


Figure 1. Transmission electron microscopy images of SWCNTs and MWCNTs.

MWCNT: Multi-walled carbon nanotube; SWCNT: Single-walled carbon nanotube.

thermal conductivity, as well as metallic or semiconducting behavior. In addition, CNTs have the capacity to cross biological barriers [7,8] and to be non-immunogenic when shortened, functionalized and rendered highly dispersible in water [9]. They can be eliminated *in vivo* via renal and/or fecal excretion. In addition, CNTs can also be exploited as new tools for modulation of molecular functions by directly affecting various biological processes or by interaction with bioactive molecules [10].

There are no comparative studies on the behavior of functionalized SWCNTs and MWCNTs in the field of biomedical applications. It is still not obvious if one type of CNTs presents more advantages than the other. Both types of CNTs are definitely attractive due to their capacity to easily translocate through cell membranes with reduced cytotoxic effects [7]. Furthermore, SWCNTs present the advantage to be detected *in vivo* because of their photoluminescence properties and can, therefore, be developed for diagnostic

purposes [11]. On the other hand, MWCNTs have a higher internal diameter than SWCNTs; the internal cavity of MWCNTs can thus be exploited for encapsulation of therapeutic agents [12]. In addition, MWCNTs have a higher external surface area that offers more possibilities of conjugation or interaction with bioactive molecules. Besides CNTs, other carbon-based nanomaterials, such as carbon nanohorns [13-16] or carbon nanodiamonds [17], are currently under investigation for therapeutic and diagnostic applications [18], but they are not treated in this review.

The aim of this review is to describe the potential of CNTs for the delivery of therapeutic agents in the context of drug discovery for innovative therapies. The strategies to render them biocompatible by functionalization with the active drugs, the possibilities for specific targeting and the options for imaging and tracking are presented. Finally, we describe the different mechanisms of cell uptake and the biodistribution, accumulation and elimination characteristics of these novel nanomaterials.

2. Drug delivery and targeting

The unique characteristics of CNTs have fascinated the scientific community and their exploitation has allowed the development of innovative strategies using CNTs in the context of drug discovery and delivery. Our laboratories were among the first to explore the properties of CNTs as vectors for drugs to treat infectious diseases [19,20]. Antibiotic amphotericin B (AmB) is one of the most effective antimycotic agents to treat chronic fungal infections. However, it is poorly soluble in water and forms aggregates, which render it highly toxic to mammalian cells. The conjugation of AmB to both SWCNTs and MWCNTs led to decreased aggregation of the drug by increasing its solubility, with resulting lower cytotoxicity. In addition, an improved efficacy of the antifungal activity was observed for AmB conjugated to CNTs by comparison with free AmB against fungal strains that are infectious against humans. This pioneering work could lead to important implications on the clinical use of AmB, whose antibiotic efficiency is currently limited by its narrow therapeutic index.

In cancer therapy, major bottlenecks include the poor specificity of chemotherapeutic drugs to reach tumor tissues, dose-dependent side effects [21] and also the limited cellular entry of various therapeutically active molecules. Nanotechnologies that involve nanoparticles [22,23], nanoshells [24], nanorods [25,26], nanowires [27] and CNTs [6] have attracted an increasing interest to develop new approaches that may offer high degree of efficacy [28-31]. The different strategies using CNTs conjugated with anticancer agents in drug discovery are summarized in Table 1.

We covalently tethered methotrexate (MTX) to MWCNTs via different linkers [5,32,33], including functional groups sensitive to intracellular enzymes. MTX is a folic acid (FA) antagonist with a potent anticancer activity, but its efficiency is hampered by a limited cell uptake and high toxic side effects.

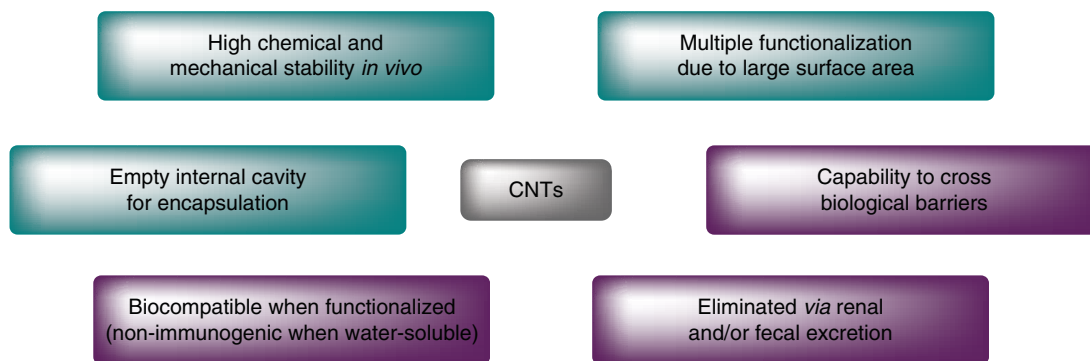


Figure 2. Properties of functionalized CNTs advantageous in drug delivery and discovery.

CNT: Carbon nanotube.

The cytotoxic activity of the MTX-MWCNT conjugates against MCF-7 cells was strongly dependent on the type of linker; in fact, MTX grafted via a tetrapeptide displayed the highest activity. We hypothesized that the enhanced anticancer action stems from the efficient intracellular enzymatic hydrolysis of MTX tethered by the cleavable peptide in comparison to a less sensitive ester bond. This work reported a promising alternative for improved cancer therapeutics of clinically-established drug molecules.

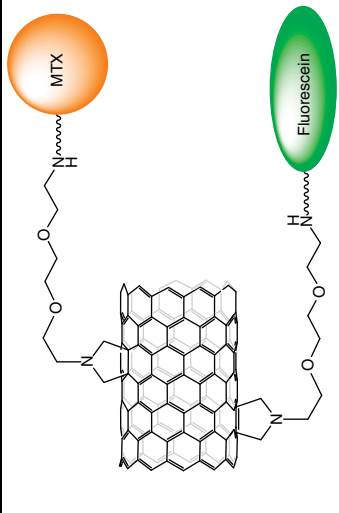
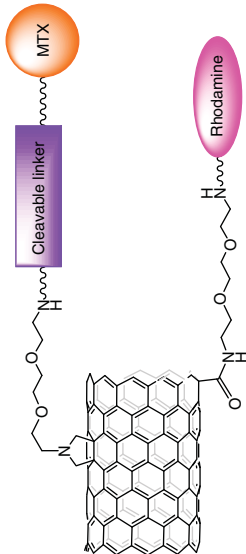
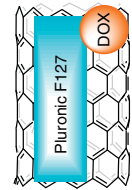
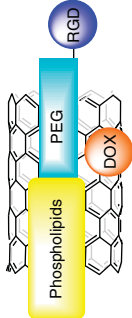
The CNT backbone has been alternatively used as a platform for the formation of supramolecular complexes with small drugs via π - π and/or hydrophobic interactions. In this context, non-covalent complexes were formed between doxorubicin (DOX) and MWCNTs previously dispersed in aqueous solution by tri-block copolymer Pluronic F127 [34]. DOX is another widely used chemotherapeutic agent, but its efficiency is limited by severe side effects. The cytotoxicity against human breast cancer cells was higher for the DOX-MWCNTs complexes than for DOX alone or DOX-surfactant preparation. The group of Dai reported similar results using SWCNTs. These nanotubes were initially functionalized either covalently with polyethylene glycol (PEG) or non-covalently with a phospholipid (PL)-PEG surfactant [35]. DOX was then loaded on the surface of CNTs and its release was found dependent on the nanotube diameter. Indeed, the release of the drug was slower when it was bound to larger diameter SWCNTs because of a higher strength of π -stacking of aromatic molecules onto larger nanotubes. Therefore, the release rate of the drug by pH control could be tailored by using SWCNTs of suitable dimensions. The DOX delivery was targeted using a cyclic RGD (Arg-Gly-Asp) peptide, introduced on the terminal groups of PEG chains. The RGD sequence binds to integrin $\alpha_v\beta_3$ receptors that are upregulated in many solid tumors [36]. Enhanced uptake of DOX by integrin $\alpha_v\beta_3$ -positive U87MG cells was observed when bound to PL-PEG-SWCNTs conjugated to RGD. Recently, Dai and co-workers expanded their work to *in vivo* studies [37]. The DOX-conjugates were tail vein injected in mice bearing Raji lymphoma xenografts. By comparison

with free DOX and DOXIL[®] (a liposomal encapsulation of DOX), DOX loaded on PL-PEG-SWCNTs showed prolonged blood circulation half-life, presumably due to PEG that attenuates the clearance by macrophages. Consequently, the uptake of these conjugates in tumor cells was enhanced, leading to a greater inhibition of tumor growth and prolonged survival compared to free DOX. However, mice treated with DOXIL exhibited a higher tumor regression, despite an increased toxicity and animal mortality.

In another study, SWCNTs were functionalized with three different moieties: i) DOX, ii) a mAb targeting the tumor marker carcinoembryonic antigen (CEA) and iii) fluorescein for imaging [38]. Antibody and fluorescein were first covalently linked to protein bovine serum albumin, which was subsequently bound to carboxylic groups of oxidized SWCNTs. DOX was then adsorbed onto the nanotube surface. This conjugate was efficiently internalized by CEA-expressing WiDr colon cancer cells. DOX was rapidly released from the nanotube surface, reaching the nucleus to exert its cytotoxic action, while SWCNTs remained in the cytoplasm. Recent *in vitro* studies reported an alternative approach for targeted delivery and pH-controlled release of DOX loaded on polysaccharide-coated SWCNTs [39]. The nanotubes were non-covalently wrapped by sodium alginate (ALG), followed by chitosan (CHI) and DOX was then loaded on the surface of the polysaccharide-modified SWCNTs. The release of DOX was triggered at slightly acidic pH, which can be found in tumor environment, as well as in lysosomes and endosomes. The authors of this study explained that the loading efficiency and release rate could be controlled by modifying the ζ potential of the functionalized SWCNTs through variation of the ALG:CHI ratio. FA, which is a targeting agent for many tumors [40], was grafted to CHI. Folate receptors are expressed at high levels on the surface of several cancer cells. The FA-conjugated SWCNTs were internalized more efficiently into HeLa cells and were more cytotoxic than free DOX and functionalized SWCNTs non-conjugated to FA.

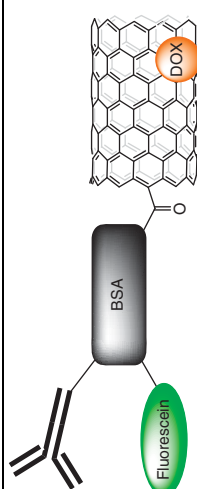
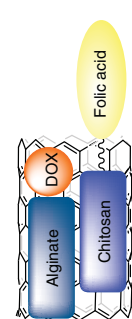
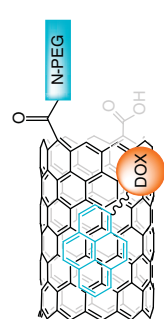
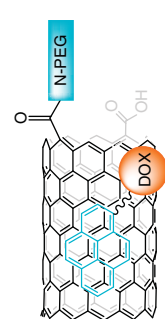
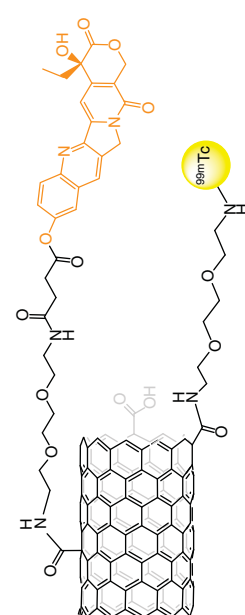
Recently, Sengupta and co-workers covalently attached DOX to pyrene by a carbamate bond, while the pyrene moiety

Table 1. Characteristics of *in vitro* and/or *in vivo* models using CNTs conjugated with anticancer agents in drug discovery.

Type of CNTs	Therapeutic agent	Targeted or non-targeted approach	Functionalization	<i>In vitro</i> models	<i>In vivo</i> models	Ref.
MWCNTs	Methotrexate	Non-targeted		Human Jurkat T lymphocytes	None	[32]
MWCNTs	Methotrexate	Non-targeted		Human breast carcinoma MCF-7	None	[33]
MWCNTs	Doxorubicin	Non-targeted		MCF-7 human breast cancer cells	None	[34]
SWCNTs	Doxorubicin	Targeted		MCF-7 breast cancer cells and U87MG human glioblastoma cancer cells	SCID mice bearing Raji lymphoma xenograft tumors	[35,37]

CNT: Carbon nanotube; Dox: Doxorubicin; EGF: Epidermal growth factor; HCPT: 10-Hydroxycamptothecin; MTX: Methotrexate; MWCNT: Multi-walled carbon nanotube; PTX: Paclitaxel; RTA: Ricin toxin A; SWCNT: Single-walled carbon nanotube.

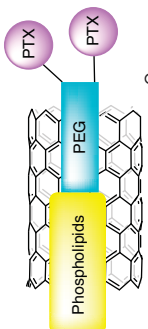
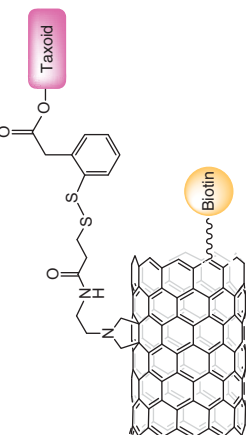
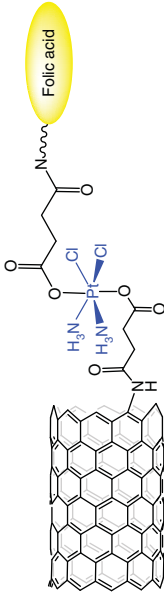
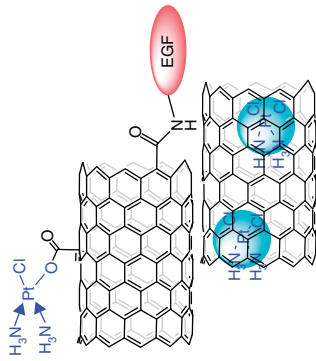
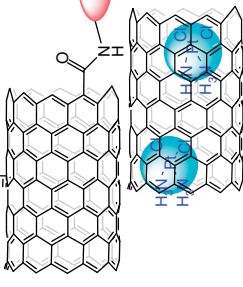
Table 1. Characteristics of *in vitro* and/or *in vivo* models using CNTs conjugated with anticancer agents in drug discovery (continued).

Type of CNTs	Therapeutic agent	Targeted or non-targeted approach	Functionalization	<i>In vitro</i> models	<i>In vivo</i> models	Ref.
SWCNTs	Doxorubicin	Targeted		WIDr human colon cancer cells	None	[38]
SWCNTs	Doxorubicin	Targeted		Human cervical carcinoma HeLa cells	None	[39]
MWCNTs	Doxorubicin	Non-targeted		B16/F10 melanoma cells	C57/BL/6 mice with B16/F10 melanoma cells implanted subcutaneously	[41]
MWCNTs	Doxorubicin	Non-targeted		HUVECs	Zebrafish embryo	[43]
MWCNTs	HCPT	Non-targeted		Human gastric carcinoma MKN-28 cells	ICR mice with H22 tumor cells inoculated subcutaneously into the armpit	[46]

CNT: Carbon nanotube; Dox: Doxorubicin; EGF: Epidermal growth factor; HCPT: 10-Hydroxycamptothecin; MITX: Methotrexate; MWCNT: Multi-walled carbon nanotube; PTX: Paclitaxel; RTA: Ricin toxin A; SWCNT: Single-walled carbon nanotube.

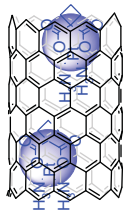
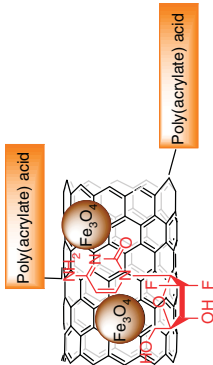
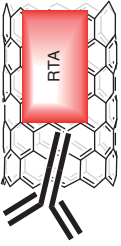
The alluring potential of functionalized carbon nanotubes in drug discovery

Table 1. Characteristics of *in vitro* and/or *in vivo* models using CNTs conjugated with anticancer agents in drug discovery (continued).

Type of CNTs	Therapeutic agent	Targeted or non-targeted approach	Functionalization	<i>In vitro</i> models	<i>In vivo</i> models	Ref.
SWCNTs	Paclitaxel	Non-targeted	 <p>The diagram shows a single-walled carbon nanotube (SWCNT) with three functional groups attached to its surface: Phospholipids (yellow box), PEG (blue box), and PTX (purple circles).</p>	4T1 murine breast cancer cell line	4T1 tumor bearing mice	[47]
SWCNTs	Taxoid	Targeted	 <p>The diagram shows a SWCNT with a Taxoid molecule (pink box) and a Biotin molecule (orange circle) attached to its surface.</p>	Murine leukemia L1210 and WI38 human lung fibroblast cell lines	None	[48]
SWCNTs	Cisplatin	Targeted	 <p>The diagram shows a SWCNT with a Cisplatin molecule (PtCl₂(NH₂)₂) and a Folic acid molecule (yellow oval) attached to its surface.</p>	Human nasopharyngeal epidermoid carcinoma (KB), choriocarcinoma (JAR) and human testicular cancer (NTera-2)	None	[51]
SWCNTs	Cisplatin	Targeted	 <p>The diagram shows a SWCNT with a Cisplatin molecule (PtCl₂(NH₂)₂) and an EGF molecule (red oval) attached to its surface.</p>	Head and neck squamous cell carcinoma (HN12 and HN13)	HN12 xenograft mice	[52]
SWCNTs	Cisplatin	Non-targeted	 <p>The diagram shows a SWCNT with a Cisplatin molecule (PtCl₂(NH₂)₂) attached to its surface.</p>	Prostate cancer cells (DU 145 and PC3 cell lines)	None	[53]

CNT: Carbon nanotube; Dox: Doxorubicin; EGF: Epidermal growth factor; HCPT: 10-Hydroxycamptothecin; MTX: Methotrexate; MWCNT: Multi-walled carbon nanotube; PTX: Paclitaxel; RTA: Ricin toxin A; SWCNT: Single-walled carbon nanotube.

Table 1. Characteristics of *in vitro* and/or *in vivo* models using CNTs conjugated with anticancer agents in drug discovery (continued).

Type of CNTs	Therapeutic agent	Targeted or non-targeted approach	Functionalization	<i>In vitro</i> models	<i>In vivo</i> models	Ref.
MWCNTs	Carboplatin	Non-targeted		Human bladder cancer cell line EJ28	None	[54]
MWCNTs	Gemcitabine	Non-targeted		None	Sprague-Dawley rats	[55]
MWCNTs	Toxin protein RTA	Targeted		L-929, HL7702, MCF-7, HeLa, and COS-7 cancer cells	None	[58]

CNT: Carbon nanotube; Dox: Doxorubicin; EGF: Epidermal growth factor; HCPT: 10-Hydroxycamptothecin; MTX: Methotrexate; MWCNT: Multi-walled carbon nanotube; PTX: Paclitaxel; RTA: Ricin toxin A; SWCNT: Single-walled carbon nanotube.

adsorbed on the nanotube surface [41]. The DOX–SWCNT conjugate induced time-dependent cell death in B16-F10 melanoma cells *in vitro*. The slow release of the drug was presumably due to the enzymatically cleavable carbamate linker, which is more stable at neutral pH than an ester, but is cleaved in the presence of cancer cell lysates, being the carboxylesterase enzyme expressed in several tumors including melanoma [42]. *In vivo* studies in a mouse B16-F10 melanoma model showed reduction in tumor growth without the systemic toxic side effects associated with free DOX. The action mechanism of DOX conjugated to carbon nanostructures was investigated in more detail by Sengupta and co-workers [43]. The activity of the DOX–SWCNT conjugate was compared to that of DOX covalently linked to polyhydroxylated fullerenes (fullerenols). Tumor inhibition was induced by both conjugates in zebrafish and murine tumor angiogenic models with opposite effects. Indeed, while DOX–SWCNTs exerted a pro-angiogenic effect *in vitro* and *in vivo*, DOX–fullerenol displayed an antiangiogenic activity. Mechanistic investigations revealed that DOX–SWCNTs attenuated the cytotoxic effect of the drug on the endothelial cells and promoted endothelial tubulogenesis, a late step during angiogenesis, whereas the fullerene-based DOX inhibited endothelial cell proliferation. This work emphasizes the critical role of the shape of nanostructures on their biological activity [44,45].

Another antitumor agent, 10-hydroxycamptothecin (HCPT), has been covalently linked to MWCNTs through a cleavable ester linkage [46]. HCPT is a potent anticancer drug that inhibits the DNA enzyme topoisomerase I, but its clinical application is hindered by low solubility in aqueous medium. The conjugation of HCPT to MWCNTs allowed increasing the water solubility, cell uptake and cancer cell cytotoxicity. When injected in tumor-bearing mice, the HCPT–MWCNT conjugate, labeled with ^{99m}Tc , exhibited a superior antitumor activity due to higher tumor accumulation and blood circulation (3.6 h) by comparison with clinical HCPT formulation.

Dai and co-workers conjugated paclitaxel (PTX) via a cleavable ester bond to branched PEG–PL chains on SWCNTs [47]. PTX is clinically used as cremophor-based formulation (commercially known as Taxol[®]) and displays reduced efficiency as it is rapidly blood cleared via renal and fecal excretion. The CNT conjugates showed higher efficacy in suppressing tumor growth than Taxol and PEGylated PTX in a 4T1 murine breast-cancer model, due to longer blood circulation and 10-fold higher drug uptake.

Ojima and co-workers functionalized SWCNTs covalently with taxoid (SB-T-1214), via a cleavable linker, and with biotin (vitamin H or vitamin B7) as targeting agent of cancer cells [48]. SB-T-1214 is a second-generation taxoid, which exhibits 2 – 3 orders of magnitude higher potency against multidrug-resistant cancer cell lines in comparison to Taxol. Taxoid was linked to SWCNTs via a disulfide bridge, which reduced its potency and, therefore, minimized its systemic toxicity in blood circulation. Specific receptor-mediated

endocytosis of the conjugate into cancer cells overexpressing biotin receptors on their surface was observed. The subsequent release of taxoid by cleavage of disulfide bond was reported by intracellular glutathione, and then the drug bound to microtubules, leading to tumor cell death.

Another potent anticancer drug that has been delivered using CNTs is cisplatin, a widely used clinical agent [49]. Different approaches have been attempted to conjugate the drug to CNTs via non-covalent interactions, covalent bonding or even encapsulation inside the nanotube internal cavity. The first approach developed by Lippard and co-workers used SWCNTs non-covalently functionalized with PL–PEG to deliver a platinum(IV) complex, a prodrug that can be intracellularly reduced to an active platinum(II) derivative [50]. The conjugate showed a substantial increase in cytotoxicity profile and a higher cell uptake compared to the free complex. By introducing FA as an axial ligand in the Pt(IV) complex, the cell-killing properties of the new conjugate were enhanced with regard to folate receptor-positive cancer cells [51]. In another study, SWCNTs were covalently functionalized with cisplatin and the epidermal growth factor (EGF) to offer specific binding to the EGF receptor, expressed aberrantly in most squamous cancer cells [52]. The higher specificity of the construct to target and kill the cancer tissues via receptor-mediated internalization was demonstrated both *in vitro* and *in vivo*, resulting in rapid decrease in tumor volume in mice compared to free cisplatin. Alternatively, cisplatin was encapsulated inside the empty inner space of SWCNTs by mixing in DMF solution [53]. This approach allowed protection of cisplatin from photodegradation and external reactive species, thus, avoiding drug decomposition. The resulting SWCNTs were coated with a PEG-lipid surfactant and the cisplatin release occurred over a period of 72 h. The conjugate was taken up by prostate cancer cell lines with a dose-dependent decrease of the cell viability. In another study, MWCNTs have been used to encapsulate carboplatin, which has higher water solubility and fewer side effects than its parent agent cisplatin [54]. The molecule was incorporated inside open-ended MWCNTs by a wet-chemical approach driven by capillarity. *In vitro* studies indicated that the filled nanotubes mediated growth inhibition of bladder cancer cells EJ28 in a dose-dependent manner, while unfilled opened MWCNTs did not affect cell viability. These studies show evidence that cisplatin-based drug conjugated to CNTs is in general more efficient to kill cancer cells than the free drug; however, improvements in the encapsulation, retention and release of the drug molecules in physiological media are needed in order to explore this technology further.

Besides developing methods to eradicate solid tumors, lymphatic metastases are also important in cancer progression. It is, therefore, crucial to develop efficient lymphatic targeted drug delivery systems to tackle metastatic cancer cells from the regional lymphatic system. For this purpose, poly(acrylic acid) grafted-MWCNTs were decorated with magnetic Fe_3O_4 nanoparticles on their outer surface of the nanotube and loaded

with the anticancer agent gemcitabine (GEM) [55]. The *in vivo* lymphatic targeting properties of GEM were investigated through subcutaneous administration in rats. The concentration of free GEM in the lymphatic system was low, while the GEM-MWCNT conjugates were readily taken up into lymph vessels by the enhanced permeability and retention (EPR) effect and delivered the drug into the lymph nodes of rats by applying an external magnetic field, which aggregates the magnetic MWCNTs at the specific location. The EPR phenomenon is due to an increased vascular permeability and a decrease in the lymphatic drainage system in tumor cells. It has been recognized as a general effect leading to the passive accumulation of macromolecular drugs in tumor cells [56,57].

MWCNTs have been also used as targeted delivery systems of other types of molecules in cancer therapy. For example, protein toxins are extremely promising but their high cytotoxicity is non-selective, thus, limiting their applications. The recombinant ricin toxin A (RTA) chain protein catalytically inactivates ribosomes, but suffers from low cell uptake capability. RTA was loaded on the surface of MWCNTs by non-specific binding [58]. The RTA-MWCNT complexes were translocated to the cytoplasm of various cell lines, causing protein synthesis inhibition and higher cell death rates compared to free RTA. Moreover, an anti-HER2 antibody, which targets HER2-overexpressing breast cancer cells, was non-covalently complexed together with RTA to MWCNTs. A statistical difference between the mortality of cancer versus normal cells was observed, indicating that the conjugate selectively destroyed the cells that overexpress HER2.

Taken together, many studies have shown the potential of functionalized CNTs as drug delivery systems to maintain and occasionally enhance the biological activity of therapeutic agents.

3. Mechanisms of cell uptake

The wide range of studies described above that attempt to explore the use of functionalized CNTs in the field of drug delivery and discovery have their origin in the capacity of CNTs to cross cell membranes [7]. Several reports have addressed the elucidation of the mechanisms of functionalized CNT cellular uptake [59]. Two main routes of internalization have been proposed: i) direct cytoplasmic translocation by insertion/diffusion and ii) uptake via phagocytosis/endocytosis processes (Figure 3). These two alternatives can be dependent on the differences in terms of CNT diameter and length [60], functional group and degree of functionalization.

The direct translocation of cell membranes by functionalized CNTs, involving insertion and passive diffusion of the nanotubes across cell membrane in a non-invasive way has been originally proposed by our laboratories [19,61,62]. Observation by TEM of ultrathin transverse sections of HeLa cells incubated with functionalized MWCNTs revealed the nanotubes able to cross the cell membrane without damaging or irreversibly disrupting this barrier [63]. We hypothesized that

the cylindrical shape, the high aspect ratio, and the semi-rigid and elongated form of CNTs allowed their penetration through the plasma membrane by a spontaneous mechanism, such as a 'nanoneedle', without causing cell death. This sort of non-classical transport activity has also been observed for some peptides and proteins [64] and theoretically predicted for nanotube-shaped objects [65]. This process was further supported by experiments where functionalized CNTs were internalized even under conditions preventing energy-dependent processes [7]. Indeed, incubation of CNTs with cells at low temperature or in the presence of an inhibitor of endosome-mediated translocation did not prevent their translocation capability. Evidence that supports the cell membrane translocation hypothesis of functionalized CNTs has also been observed by others [66]. In addition, Cai *et al.* proposed CNT sparring of mammalian cells for molecular delivery guided by a magnetic field to cross the cell membrane and intracellularly translocate their cargo [67]. This work aimed to demonstrate that it is possible to induce a temporary permeabilization of cell membranes without perturbation.

Internalization processes based on phagocytosis [68] or endocytosis [69-73] have been elucidated by various research groups. In particular, Dai and co-workers reported that both SWCNTs covalently linked to proteins [8,74] or non-covalently coated with DNA [74] were internalized via an endocytotic mechanism. Their uptake was blocked at 4°C and in the presence of endocytosis inhibitors. Endocytosis occurs through clathrin-coated pits, rather than through caveolae or lipid-raft routes. It is rather difficult to fully discriminate between the different possible mechanisms of internalization. Indeed, incubation temperature should be carefully considered as it can affect not only endocytotic processes, but also membrane piercing by CNTs as the fluidity of cell membranes is strongly dependent on temperature. Thus, a decrease of CNT cellular uptake observed at 4°C does not necessarily involve an endocytosis-like mechanism, unless it is corroborated by further evidence. The concomitant existence of multiple possible mechanisms of cell uptake can be attributed to significant differences in terms of both the nanotube material (i.e., type of functionalization and size of CNTs) and the experimental procedures (particularly cell types).

Surface functionalization-dependent internalization of SWCNTs by cells has been also observed [75]. SWCNTs coated with short PEG₂₀₀₀ exhibited higher cell uptake compared to SWCNTs coated with longer PEG₅₄₀₀. This difference probably derives from more exposed hydrophobic surfaces in the case of SWCNTs coated with shorter PEG, due to incomplete coverage of the nanotube sidewall. The retained hydrophobicity can interact with hydrophobic cell membrane domains, resulting in binding and association with cells, which can lead to cell internalization via endocytosis. In another study, prolonged sonication of PL-PEG₂₀₀₀-wrapped SWCNTs led to PEG fragmentation [76]. When the PEG integrity was preserved, the coating prevented the nonspecific uptake of SWCNTs by cells, while nanotubes

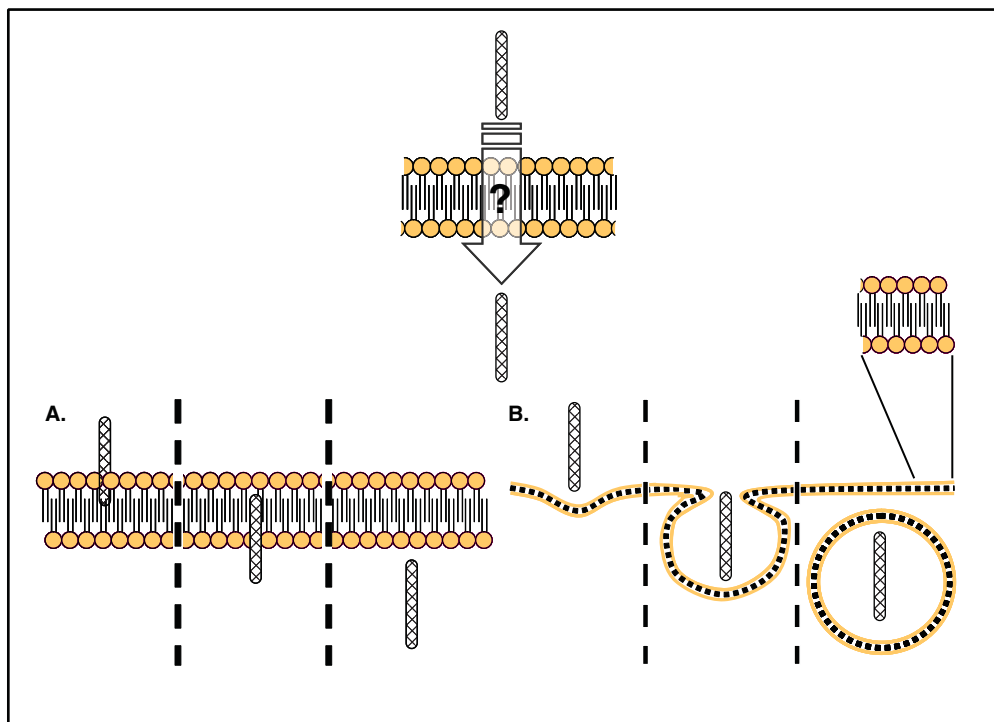


Figure 3. The ‘nanoneedle’ (A.) and endocytotic (B.) cellular internalization mechanisms.

were internalized when PEG was fragmented. Although the authors did not speculate on possible uptake mechanisms, these findings underline the importance of CNT coating in determining their biological fate.

Once internalized in cells, SWCNTs have been described to be able to exit cells through exocytosis. The group of Strano reported a study using DNA-wrapped SWCNTs tracked during *in vitro* trafficking by exploiting their intrinsic near infrared fluorescence properties [77]. These authors hypothesized that the DNA coating of the SWCNTs could lead to a subsequent clustering in aggregates, which would be significant enough to reach the minimum radius that is required thermodynamically to allow endocytosis [78,79]. Co-localization experiments showed SWCNTs inside lysosomes, confirming endocytotic uptake. Once inside the cells, SWCNTs were mainly confined inside vesicles, until they were expelled by the cells by exocytosis. In an intriguing study by a different group, reversible accumulation of PEGylated SWCNTs was observed *in vitro*. Fluorescently labeled PEG–SWCNTs accumulated into the nucleus, in particular the nucleolus [80]. When the nanotubes were removed from the cell culture medium, the internalized CNTs rapidly moved to the nucleus, then moved out of the nucleus and were eventually released from the cells. These results suggested that the translocation of PEGylated SWCNTs through the plasma membrane and nuclear envelope can be bi-directional.

Overall, these experiments highlight the possibility of various mechanisms of cell uptake, which strongly depend

on the nanotube types used and their surface functionalization. The different mechanisms can be exploited to target different cell compartments and to modulate the activity of various drug molecules either covalently linked or adsorbed on the nanotubes.

4. Pharmacokinetic studies

The other fundamental aspect related to the development of a novel system for drug delivery concerns the evaluation of their pharmacokinetic characteristics. Similar to cell penetration, the biodistribution studies of functionalized CNTs are affected by many different variables including the techniques of analysis, the types of nanotubes and the differences in functionalization. Moreover, the plethora of animal models and the different administration routes play a critical role and make comparison of results difficult to interpret. A general agreement on acute toxicity is beginning to emerge based on all published studies that report no adverse events if low concentrations of short CNTs are used. However, it is beyond the scope of this review to address the general issue of toxicity associated with the different types of CNTs, especially those that are not chemically functionalized and not developed for biomedical applications [81–84]. The different strategies using functionalized CNTs for blood circulation and biodistribution studies are summarized in Table 2.

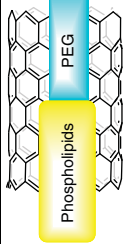
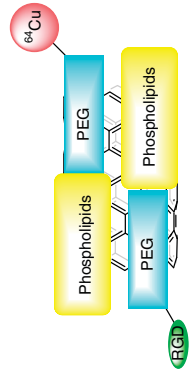
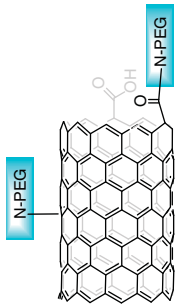
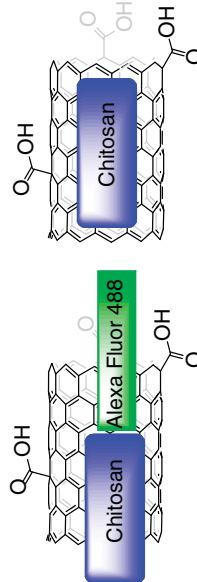
One of the first studies on biodistribution and clearance of CNTs was reported by our laboratories, where a radionuclide

Table 2. Characteristics of blood circulation and biodistribution studies using functionalized CNTs.

Type of CNTs	Functionalization	Characterization	<i>In vivo</i> models	Admin. route	Blood clearance half-life	Biodistribution	Ref.
SWCNTs/ MWCNTs		Radiolabeling (¹¹¹ In)	Mice	i.v.	3 – 3.5 h	Kidney, bladder	[85]
MWCNTs		Radiolabeling (¹¹¹ In)	Rat	i.v.	ND	Kidney, bladder	[86]
SWCNTs		Radiolabeling (^{86y} Tc and ¹¹¹ In)	Mice	i.v., i.p. and retroorbital sinus injection	< 3 h (^{86y} Tc retroorbital sinus injection)	Kidney, spleen, liver, bone	[89]
MWCNTs		Radiolabeling (^{99m} Tc)	Mice	i.p.	5.5 h	Enterogastric area, stomach	[90]

CNT: Carbon nanotube; i.p.: Intraperitoneal; i.v.: Intravenous; MWCNT: Multi-walled carbon nanotube; SWCNT: Single-walled carbon nanotube.

Table 2. Characteristics of blood circulation and biodistribution studies using functionalized CNTs (continued).

Type of CNTs	Functionalization	Characterization	In vivo models	Admin. route	Blood clearance half-life	Biodistribution	Ref.
SWCNTs		Raman spectroscopy	Mice	i.v.	ND	Liver, spleen	[91]
SWCNTs		Radiolabeling (⁶⁴ Cu) and Raman spectroscopy	Tumor-bearing mice	i.v.	2 h (PEG ₅₄₀₀ -SWCNT), 0.5 h (PEG ₂₀₀₀ -SWCNT)	Liver, tumor	[92]
SWCNTs		Isotope ratio mass spectrometry (¹³ C)	Tumor-bearing mice	i.v.	15.3 h	Liver, spleen, tumor	[93]
SWCNTs		Fluorescence and Raman spectroscopy	Mice	i.v.	3 – 4 h	Liver, spleen, kidney	[94]

CNT: Carbon nanotube; i.p.: Intraperitoneal; i.v.: Intravenous; MWCNT: Multi-walled carbon nanotube; SWCNT: Single-walled carbon nanotube.

was linked to both SWCNTs and MWCNTs [85]. The covalent functionalization of CNTs by means of 1,3-dipolar cycloaddition allowed the introduction of diethylenetriamine-pentaacetic acid (DTPA) to chelate ^{111}In . The concentration in kidney was very high after 30 min, meaning a preferential tropism for that organ and a fast clearance from the blood stream without accumulation in the other organs. The fast elimination was also confirmed by the low quantity of radioactivity detected 3 h after the injection, with a blood circulation half-life of about 3 – 3.5 h. Analogous conjugates, built on MWCNTs, were also used for similar studies in rats [86]. The labeled constructs were later injected into the tail vein and their distribution was followed by microsingle photon emission tomography. Rapid accumulation in kidneys and bladder was observed with almost the total amount of labeled compounds excreted into urine within 6 h. Again, no radioactivity was detected in lungs or the organs of the reticulo-endothelial system. The behavior of DTPA-functionalized MWCNTs without ^{111}In was compared to the distribution of simply purified MWCNTs. Histological analyses showed non-functionalized nanotubes accumulated into Kupffer cells, while no traces of DTPA-MWCNTs were found in lung or liver [87].

The mechanism of renal clearance was thoroughly investigated, indicating that the longitudinal dimension of functionalized CNTs does not represent a problem for the glomerular filtration if the nanotubes perpendicularly approach the fenestration membrane [88]. The reported renal clearance was also confirmed independently by McDevitt *et al.* who linked the chelating agent DOTA to SWCNTs functionalized by 1,3-dipolar cycloaddition for the chelation of ^{86}Y and ^{111}In [89]. Two groups of mice were intravenously (i.v.) and intraperitoneally (i.p.) injected with the ^{86}Y -SWCNT derivatives, respectively. Kidney images showed uptake in the renal cortex but not in the medulla. On the other hand, a considerable percentage of radioactivity was measured in liver and spleen, with a concentration dependent on the method of administration. Clearance studies were performed using positron emission tomography and the ^{111}In -DOTA-SWCNT derivatives were injected via the retroorbital sinus. Urinary clearance was found to be taking place rapidly, with retention of only a low percentage of injected dose after 20 h.

Guo *et al.* functionalized MWCNTs with glucosamine to complex $^{99\text{m}}\text{Tc}$ [90]. The tissue distribution was analyzed at short times after i.p. injection with evident retention of the complex in the gastrointestinal district and in the stomach, although excretion was quite fast (5.5 h) and only traces of radioactivity were found in the entire body. Excretion took place mainly by the bile and fecal routes but a considerable percentage of radioactivity was measured also in the urine.

In another study using different nanotube material, short SWCNTs were non-covalently functionalized using PLs conjugated to linear or branched PEG units [91]. Their blood circulation was strongly influenced by the PEG construct, with an increase from 1.2 to 15 h ranging from PEG₂₀₀₀ to longer

and more branched PEG units, confirming the results reported in a previous article by the same authors [92]. This evidence allows hypothesizing that the branched PEG chains protect and shield CNTs affording biological inertness. Although the main uptake is ascribed to liver and spleen, the presence of high molecular mass PEG (i.e., 5 and 7 kDa) delayed accumulation in these organs. Interestingly, 1 day after the administration of PEG₅₀₀₀-SWCNTs, the Raman signals associated with CNTs were detected also in bone, kidney, intestine, stomach and lung. Over a 3-month period, the presence of SWCNTs in the liver and spleen slowly decreased with the excretion mainly via the fecal route. A small percentage of these PEG-coated nanotubes was detected in urine suggesting that only the shortest conjugates (< 50 nm in length) followed this elimination route.

Sun and co-workers found a longer circulation half-time (15.3 h) for SWCNTs covalently functionalized with PEG₁₅₀₀ following i.v. injection in tumor-bearing mice [93]. Preliminary results showed higher tumor uptake, while no significant uptake of PEG₁₅₀₀-SWCNTs was observed in the brain, intestine, muscle and stomach, in contrast to the high accumulation in liver and spleen, even after 7 days of exposure. Alternatively, Kang *et al.* used SWCNTs complexed with CHI labeled with fluorescent Alexa Fluor488 [94]. The conjugates were injected i.v. in mice. The distribution did not show remarkable differences with respect to covalently functionalized SWCNT derivatives previously reported, with preferential accumulation mainly in the liver as well as in spleen and kidney, even though to a lower extent. Other tissues presented low amount of SWCNTs but the study was performed using mainly Raman spectroscopy which presents limitations in terms of accurate and quantitative determination of nanotube concentration in organs. Based on the reported clearance kinetics, it emerged that the blood circulation half-life was around 3 – 4 h. After 24 h, almost 50% of the dose was excreted via the renal route. The fluorescence analysis evidenced the presence of granules rich in nanotubes in the liver, with injury of macrophages and cellular swelling, and also blood coagulation in the blood vessels.

Taken together, most studies on tissue biodistribution and blood kinetics today have indicated that surface modified (chemically or non-covalently) CNTs are eliminated into urine or feces with low residual amounts remaining in the tissues. The blood clearance half-life is generally in the order of hours and seems to be greatly dependent on the characteristics of CNTs and the functional groups present on the nanotube surface. The data reported today suggest that CNTs can be suitable potential nanovectors for drug delivery purposes in the absence of adverse effects.

5. Conclusions

This review describes the potential applications of functionalized CNTs in the field of drug discovery and delivery. It particularly focuses on the use of CNTs as novel platforms

for cancer therapy. Targeted methodologies are currently explored to improve the efficacy of CNT-based conjugates. The discovery of CNTs as new nanomedicine tools received an impetus because of the capacity of such nanomaterials to penetrate into the cells. The mechanisms are various and we have attempted to explain their differences and the role of the functional groups in determining one mechanism in favor of another. Finally, the pharmacokinetics properties have been described taking into account the impact and potential risks of functionalized CNTs on health following *in vivo* administration.

6. Expert opinion

The explosive development of novel nanomaterials in the last decade has offered a new rich toolbox for pharmaceutical and biomedical scientists that seek their translation to the clinical realm. One such type of nanomaterial is the CNT that offers a high surface area, hollow cylindrical platform for the attachment, coating and filling with therapeutic and diagnostic agents. The transformation of this molecular platform into a viable pharmaceutical entity is considered unique in drug discovery, as no structurally fibrillar nanoscale component currently exists as a pharmaceutical. Moreover, control of the apparently inherent capability of CNTs to directly translocate cellular membranes will offer a powerful technology for the intracellular transport of molecules that is currently lacking. It is well recognized today that paradigm-shifting advances in drug delivery development will immediately benefit the discovery of new therapeutic modalities and rejuvenate drug discovery pipelines. Developments in CNT drug delivery platforms can potentially offer this.

At the basic level of understanding CNT interactions with human cells and their behavior following CNT administration in living animals, very interesting new mechanisms have been proposed by our laboratories a few years ago that have now been the topic of very constructive and active scientific discourse in the field. At the cellular level, we proposed the occurrence of direct translocation of nanotubes through the cell membrane, termed by some as the 'nanoneedle' mechanism. A few groups have discussed this proposed mechanism through their own experiments and the overall evidence today suggests that direct translocation can indeed occur; however, other cell internalization mechanisms such as endocytosis and phagocytosis can become dominant based on the type of surface modification and length of the CNTs as well as the cell type and experimental conditions (duration of interaction, dispersing media, etc.) used in each laboratory. There is still more work required to elucidate the details of the 'nanoneedle' mechanism of CNT cell internalization and, more importantly, to find ways to control and utilize it for

biomedical applications. At the pharmacokinetic and tissue distribution level, we have also proposed (somewhat controversially) that chemically functionalized, de-bundled (i.e., non-aggregated) CNTs following intravenous administration were able to cross the glomerular filtration barrier rapidly and translocate from the blood into the urinary compartment intact and without any damage to the kidney. This proposal has also been reproduced independently by a few others using the same or similar chemical functionalization strategies; however, other studies using different types of CNTs (in particular when nanotubes are coated – non-covalently – with large molecular mass lipid and/or polymer molecules) have shown predominant accumulation in the liver tissue of i.v. injected animals. Again, we believe that this is another illustration of the importance of surface modification on the CNT material used. More systematic studies exploring the pharmacokinetic profiles of different chemically functionalized nanotubes are needed in order to control the degree of their urinary excretion depending on the desired application.

The exploration of CNTs as drug delivery systems is still at its infancy and the variability in the results reported in the literature to date is a testament for this immaturity. Nevertheless, the attractive features of CNTs can in principle offer advantages over other established delivery systems (such as liposomes, polymeric nanoparticles or matrices). As has been illustrated in this review, different types of CNTs have been reported by various laboratories to be able to transport and release a range of therapeutic agents by maintaining (and occasionally improving) biological activity. The field of oncology is the one where most such nanotube-based constructs are being developed and almost all reported studies are proof-of-principle investigations. More preclinical studies, cancer disease models and thorough systematic efficacy studies using the different types of CNT material are needed to determine their advantages and limitations. Efficacy of new therapeutic interventions to achieve cancer treatment in comparison to currently available therapy 'gold standard' or established delivery system technologies (either successful or not) is the critical factor that will determine further development of nanotube-based pharmaceuticals. Future studies that focus on the therapeutic efficacy of such constructs should attempt to address this by incorporation of the appropriate controls.

Declaration of interest

The authors are supported by the CNRS, the School of Pharmacy, the University of London, the University of Trieste and the Italian Ministry of Education MIUR (Firb RBIN04HC3S). This work was partly supported by the European Union FP6 NEURONANO (NMP4-CT-2006-031847) and FP7 ANTICARB (HEALTH-2007-201587) programs.

Bibliography

1. Monthioux M, Kuznetsov VL. Who should be given the credit for the discovery of carbon nanotubes? *Carbon* 2006;44(9):1621-3
2. Iijima S. Helical microtubules of graphitic carbon. *Nature* 1991;354(6348):56-8
3. Ajayan PM, Tour JM. Materials science: nanotube composites. *Nature* 2007;447(7148):1066-8
4. Lu F, Gu L, Meziani MJ, et al. Advances in bioapplications of carbon nanotubes. *Adv Mater* 2009;21(2):139-52
5. Prato M, Kostarelos K, Bianco A. Functionalized carbon nanotubes in drug design and discovery. *Acc Chem Res* 2008;41(1):60-8
6. Kostarelos K, Bianco A, Prato M. Promises, facts and challenges for carbon nanotubes in imaging and therapeutics. *Nat Nanotechnol* 2009;4(10):627-33
7. Kostarelos K, Lacerda L, Pastorin G, et al. Cellular uptake of functionalized carbon nanotubes is independent of functional group and cell type. *Nat Nanotechnol* 2007;2(2):108-13
8. Kam NW, Dai H. Carbon nanotubes as intracellular protein transporters: generality and biological functionality. *J Am Chem Soc* 2005;127(16):6021-6
9. Dumortier H, Lacotte S, Pastorin G, et al. Functionalized carbon nanotubes are non-cytotoxic and preserve the functionality of primary immune cells. *Nano Lett* 2006;6(7):1522-8
10. Ménard-Moyon C, Kostarelos K, Prato M, Bianco A. Functionalized carbon nanotubes for probing and modulating molecular functions. *Chem Biol* 2010;17(2):107-15
11. Bachilo SM, Strano MS, Kittrell C, et al. Structure-assigned optical spectra of single-walled carbon nanotubes. *Science* 2002;298(5602):2361-6
12. Ren Y, Pastorin G. Incorporation of hexamethylmelamine inside capped carbon nanotubes. *Adv Mater* 2008;20(11):2031-6
13. Ajima K, Maigné A, Yudasaka M, Iijima S. Optimum hole-opening condition for cisplatin incorporation in single-wall carbon nanohorns and its release. *J Phys Chem B* 2006;110(39):19097-9
14. Xu J, Yudasaka M, Kouraba S, et al. Single wall carbon nanohorn as a drug carrier for controlled release. *Chem Phys Lett* 2008;461(4-6):189-92
15. Murakami T, Sawada H, Tamura G, et al. Water-dispersed single-wall carbon nanohorns as drug carriers for local cancer chemotherapy. *Nanomedicine* 2008;3(4):453-63
16. Miyawaki J, Matsumura S, Yuge R, et al. Biodistribution and ultrastructural localization of single-walled carbon nanohorns determined in vivo with embedded Gd₂O₃ labels. *ACS Nano* 2009;3(6):1399-406
17. Xing Y, Dai L. Nanodiamonds for nanomedicine. *Nanomedicine* 2009;4(2):207-18
18. Bianco A, Kostarelos K, Prato M. Opportunities and challenges of carbon-based nanomaterials for cancer therapy. *Expert Opin Drug Deliv* 2008;5(3):331-42
19. Wu W, Wieckowski S, Pastorin G, et al. Targeted delivery of amphotericin B to cells by using functionalized carbon nanotubes. *Angew Chem Int Ed* 2005;44(39):6358-62
20. Bianco A. Carbon nanotubes for the delivery of therapeutic molecules. *Expert Opin Drug Deliv* 2004;1(1):57-65
21. Allen TM, Cullis PR. Drug delivery systems: entering the mainstream. *Science* 2004;303(5665):1818-22
22. O'Neal DP, Hirsch LR, Halas NJ, et al. Photo-thermal tumor ablation in mice using near infrared-absorbing nanoparticles. *Cancer Lett* 2004;209(2):171-6
23. Brannon-Peppas L, Blanchette JO. Nanoparticle and targeted systems for cancer therapy. *Adv Drug Deliv Rev* 2004;56(11):1649-59
24. Loo C, Lowery A, Halas N, et al. Immunotargeted nanoshells for integrated cancer imaging and therapy. *Nano Lett* 2005;5(4):709-11
25. Huang X, El-Sayed IH, Qian W, El-Sayed MA. Cancer cell imaging and photothermal therapy in the near-infrared region by using gold nanorods. *J Am Chem Soc* 2006;128(6):2115-20
26. Huff TB, Tong L, Zhao Y, et al. Hyperthermic effects of gold nanorods on tumor cells. *Nanomedicine* 2007;2(1):125-32
27. Kim W, Ng JK, Kunitake ME, et al. Interfacing silicon nanowires with mammalian cells. *J Am Chem Soc* 2007;129(23):7228-9
28. Ferrari M. Cancer nanotechnology: opportunities and challenges. *Nat Rev Cancer* 2005;5(3):161-71
29. Peer D, Karp JM, Hong S, et al. Nanocarriers as an emerging platform for cancer therapy. *Nat Nanotechnol* 2007;2(12):751-60
30. Wang X, Yang L, Chen ZG, Shin DM. Application of nanotechnology in cancer therapy and imaging. *CA Cancer J Clin* 2008;58(2):97-110
31. Farokhzad OC, Langer R. Impact of nanotechnology on drug delivery. *ACS Nano* 2009;3(1):16-20
32. Pastorin G, Wu W, Wieckowski S, et al. Double functionalization of carbon nanotubes for multimodal drug delivery. *Chem Commun* 2006;(11):1182-4
33. Samorì C, Ali-Boucetta H, Sainz R, et al. Enhanced anticancer activity of multi-walled carbon nanotube-methotrexate conjugates using cleavable linkers. *Chem Commun* 2010;46(9):1494-6
34. Ali-Boucetta H, Al-Jamal KT, McCarthy D, et al. Multiwalled carbon nanotube-doxorubicin supramolecular complexes for cancer therapeutics. *Chem Commun* 2008;(4):459-61
35. Liu Z, Sun XM, Nakayama-Ratchford N, Dai HJ. Supramolecular chemistry on water-soluble carbon nanotubes for drug loading and delivery. *ACS Nano* 2007;1(1):50-6
36. Mizejewski GJ. Role of integrins in cancer: survey of expression patterns. *Proc Soc Expert Biol Med* 1999;222(2):124-38
37. Liu Z, Fan AC, Rakhra K, et al. Supramolecular stacking of doxorubicin on carbon nanotubes for in vivo cancer therapy. *Angew Chem Int Ed* 2009;48(41):7668-72
38. Heister E, Neves V, Tilmaciu C, et al. Triple functionalisation of single-walled carbon nanotubes with doxorubicin,

The alluring potential of functionalized carbon nanotubes in drug discovery

- a monoclonal antibody, and a fluorescent marker for targeted cancer therapy. *Carbon* 2009;47(9):2152-60
39. Zhang X, Meng L, Lu Q, et al. Targeted delivery and controlled release of doxorubicin to cancer cells using modified single wall carbon nanotubes. *Biomaterials* 2009;30(30):6041-7
 40. Sudimack J, Lee RJ. Targeted drug delivery via the folate receptor. *Adv Drug Deliv Rev* 2000;41(2):147-62
 41. Chaudhuri P, Soni S, Sengupta S. Single-walled carbon nanotube-conjugated chemotherapy exhibits increased therapeutic index in melanoma. *Nanotechnology* 2010;21(2):025102 (11pp)
 42. Xu G, Zhang W, Ma MK, McLeod HL. Human carboxylesterase 2 is commonly expressed in tumor tissue and is correlated with activation of irinotecan. *Clin Cancer Res* 2002;8(8):2605-11
 43. Chaudhuri P, Harfouche R, Soni S, et al. Shape effect of carbon nanovectors on angiogenesis. *ACS Nano* 2010;4(1):574-82
 44. Geng Y, Dalhaimer P, Cai S, et al. Shape effects of filaments versus spherical particles in flow and drug delivery. *Nat Nanotechnol* 2007;2(4):249-55
 45. Champion JA, Katare YK, Mitragotri S. Particle shape: a new design parameter for micro- and nanoscale drug delivery carriers. *J Control Release* 2007;121(1-2):3-9
 46. Wu W, Li R, Bian X, et al. Covalently combining carbon nanotubes with anticancer agent: preparation and antitumor activity. *ACS Nano* 2009;3(9):2740-50
 47. Liu Z, Chen K, Davis C, et al. Drug delivery with carbon nanotubes for in vivo cancer treatment. *Cancer Res* 2008;68(16):6652-60
 48. Chen J, Chen S, Zhao X, et al. Functionalized single-walled carbon nanotubes as rationally designed vehicles for tumor-targeted drug delivery. *J Am Chem Soc* 2008;130(49):16778-85
 49. Jung Y, Lippard SJ. Direct cellular responses to platinum-induced DNA damage. *Chem Rev* 2007;107(5):1387-407
 50. Feazell RP, Nakayama-Ratchford N, Dai H, Lippard SJ. Soluble single-walled carbon nanotubes as longboat delivery systems for platinum(IV) anticancer drug design. *J Am Chem Soc* 2007;129(27):8438-9
 51. Dhar S, Liu Z, Thomale J, et al. Targeted single-wall carbon nanotube-mediated Pt(IV) prodrug delivery using folate as a homing device. *J Am Chem Soc* 2008;130(34):11467-76
 52. Bhirde AA, Patel V, Gavard J, et al. Targeted killing of cancer cells in vivo and in vitro with EGF-directed carbon nanotube-based drug delivery. *ACS Nano* 2009;3(2):307-16
 53. Tripisciano C, Kraemer K, Taylor A, Borowiak-Palen E. Single-wall carbon nanotubes based anticancer drug delivery system. *Chem Phys Lett* 2009;478(4-6):200-5
 54. Hampel S, Kunze D, Haase D, et al. Carbon nanotubes filled with a chemotherapeutic agent: a nanocarrier mediates inhibition of tumor cell growth. *Nanomedicine* 2008;3(2):175-82
 55. Yang D, Yang F, Hu J, et al. Hydrophilic multi-walled carbon nanotubes decorated with magnetite nanoparticles as lymphatic targeted drug delivery vehicles. *Chem Commun* 2009;(29):4447-9
 56. Maeda H, Wu J, Sawa T, et al. Tumor vascular permeability and the EPR effect in macromolecular therapeutics: a review. *J Control Release* 2000;65(1-2):271-84
 57. Maeda H, Seymour LW, Miyamoto Y. Conjugates of anticancer agents and polymers: advantages of macromolecular therapeutics in vivo. *Bioconjug Chem* 1992;3(5):351-62
 58. Weng X, Wang M, Ge J, et al. Carbon nanotubes as a protein toxin transporter for selective HER2-positive breast cancer cell destruction. *Mol Biosyst* 2009;5(10):1224-31
 59. Lacerda L, Raffa S, Prato M, et al. Cell-penetrating CNTs for delivery of therapeutics. *Nano Today* 2007;2(6):38-43
 60. Raffa V, Ciofani G, Nitodas S, et al. Can the properties of carbon nanotubes influence their internalization by living cells? *Carbon* 2008;46(12):1600-10
 61. Pantarotto D, Partidos CD, Hoebeke J, et al. Immunization with peptide-functionalized carbon nanotubes enhances virus-specific neutralizing antibody responses. *Chem Biol* 2003;10(10):961-6
 62. Pantarotto D, Briand JP, Prato M, Bianco A. Translocation of bioactive peptides across cell membranes by carbon nanotubes. *Chem Commun* 2004;(1):16-7
 63. Pantarotto D, Singh R, McCarthy D, et al. Functionalized carbon nanotubes for plasmid DNA gene delivery. *Angew Chem Int Ed* 2004;43(39):5242-6
 64. Kuelz LA, Middaugh CR. Nonclassical transport proteins and peptides: an alternative to classical macromolecule delivery systems. *J Pharm Sci* 2003;92(9):1754-72
 65. Lopez CF, Nielsen SO, Moore PB, Klein ML. Understanding nature's design for a nanosyringe. *Proc Natl Acad Sci USA* 2004;101(13):4431-4
 66. Lu Q, Moore JM, Huang G, et al. RNA polymer translocation with single-walled carbon nanotubes. *Nano Lett* 2004;4(12):2473-7
 67. Cai D, Mataraza JM, Qin ZH, et al. Highly efficient molecular delivery into mammalian cells using carbon nanotube spearing. *Nat Methods* 2005;2(6):449-54
 68. Cherukuri P, Bachilo SM, Litovsky SH, Weisman RB. Near-infrared fluorescence microscopy of single-walled carbon nanotubes in phagocytic cells. *J Am Chem Soc* 2004;126(48):15638-9
 69. Kam NW, Jessop TC, Wender PA, Dai H. Nanotube molecular transporters: internalization of carbon nanotube-protein conjugates into mammalian cells. *J Am Chem Soc* 2004;126(22):6850-1
 70. Kam NW, O'Connell M, Wisdom JA, Dai H. Carbon nanotubes as multifunctional biological transporters and near-infrared agents for selective cancer cell destruction. *Proc Natl Acad Sci USA* 2005;102(33):11600-5
 71. Heller DA, Baik S, Eurell TE, Strano MS. Single-walled carbon nanotube spectroscopy in live cells: towards long-term labels and optical sensors. *Adv Mater* 2005;17(23):2793-9
 72. Chin SF, Baughman RH, Dalton AB, et al. Amphiphilic helical peptide enhances the uptake of single-walled carbon nanotubes by living cells. *Exp Biol Med* 2007;232(9):1236-44
 73. Cato MH, D'Annibale F, Mills DM, et al. Cell-type specific and cytoplasmic targeting of PEGylated carbon

- nanotube-based nanoassemblies. *J Nanosci Nanotechnol* 2008;8(5):2259-69
74. Kam NW, Liu Z, Dai H. Carbon nanotubes as intracellular transporters for proteins and DNA: an investigation of the uptake mechanism and pathway. *Angew Chem Int Ed* 2006;45(4):577-81
75. Liu Z, Winters M, Holodniy M, Dai H. siRNA delivery into human T cells and primary cells with carbon-nanotube transporters. *Angew Chem Int Ed* 2007;46(12):2023-7
76. Zeineldin R, Al-Haik M, Hudson LG. Role of polyethylene glycol integrity in specific receptor targeting of carbon nanotubes to cancer cells. *Nano Lett* 2009;9(2):751-7
77. Jin H, Heller DA, Strano MS. Single-particle tracking of endocytosis and exocytosis of single-walled carbon nanotubes in NIH-3T3 cells. *Nano Lett* 2008;8(6):1577-85
78. Jin H, Heller DA, Sharma R, Strano MS. Size-dependent cellular uptake and expulsion of single-walled carbon nanotubes: single particle tracking and a generic uptake model for nanoparticles. *ACS Nano* 2009;3(1):149-58
79. Gao H, Shi W, Freund LB. Mechanics of receptor-mediated endocytosis. *Proc Natl Acad Sci USA* 2005;102(27):9469-74
80. Cheng J, Fernando KA, Veca LM, et al. Reversible accumulation of PEGylated single-walled carbon nanotubes in the mammalian nucleus. *ACS Nano* 2008;2(10):2085-94
81. Ren HX, Chen X, Liu JH, et al. Toxicity of single-walled carbon nanotube: how we were wrong? *Mater Today* 2010;13(1-2):6-8
82. Boczkowski J, Lanone S. Potential uses of carbon nanotubes in the medical field: how worried should patients be? *Nanomedicine* 2007;2(4):407-10
83. Poland CA, Duffin R, Kinloch I, et al. Carbon nanotubes introduced into the abdominal cavity of mice show asbestos-like pathogenicity in a pilot study. *Nat Nanotechnol* 2008;3(7):423-8
84. Ryman-Rasmussen JP, Cesta MF, Brody AR, et al. Inhaled carbon nanotubes reach the subpleural tissue in mice. *Nat Nanotechnol* 2009;4(11):747-51
85. Singh R, Pantarotto D, Lacerda L, et al. Tissue biodistribution and blood clearance rates of intravenously administered carbon nanotube radiotracers. *Proc Natl Acad Sci USA* 2006;103(9):3357-62
86. Lacerda L, Soundararajan A, Singh R, et al. Dynamic imaging of functionalized multi-walled carbon nanotube systemic circulation and urinary excretion. *Adv Mater* 2008;20(2):225-30
87. Lacerda L, Ali-Boucetta H, Herrero MA, et al. Tissue histology and physiology following intravenous administration of different types of functionalized multiwalled carbon nanotubes. *Nanomedicine* 2008;3(2):149-61
88. Lacerda L, Herrero MA, Venner K, et al. Carbon-nanotube shape and individualization critical for renal excretion. *Small* 2008;4(8):1130-2
89. McDevitt MR, Chattopadhyay D, Jaggi JS, et al. PET imaging of soluble yttrium-86-labeled carbon nanotubes in mice. *PLoS One* 2007;2(9):e907
90. Guo J, Zhang X, Li Q, Li W. Biodistribution of functionalized multiwall carbon nanotubes in mice. *Nucl Med Biol* 2007;34(5):579-83
91. Liu Z, Davis C, Cai W, et al. Circulation and long-term fate of functionalized, biocompatible single-walled carbon nanotubes in mice probed by Raman spectroscopy. *Proc Natl Acad Sci USA* 2008;105(5):1410-5
92. Liu Z, Cai W, He L, et al. In vivo biodistribution and highly efficient tumour targeting of carbon nanotubes in mice. *Nat Nanotechnol* 2007;2(1):47-52
93. Yang ST, Fernando KA, Liu JH, et al. Covalently PEGylated carbon nanotubes with stealth character in vivo. *Small* 2008;4(7):940-4
94. Kang B, Yu D, Dai Y, et al. Biodistribution and accumulation of intravenously administered carbon nanotubes in mice probed by Raman spectroscopy and fluorescent labeling. *Carbon* 2009;47(4):1189-92

Affiliation

Cécilia Ménard-Moyon¹, Enrica Venturelli¹, Chiara Fabbro², Cristian Samorì¹, Tatiana Da Ros², Kostas Kostarelos³, Maurizio Prato² & Alberto Bianco^{*1}
[†]Author for correspondence
¹CNRS, Institut de Biologie Moléculaire et Cellulaire, UPR 9021 Immunologie et Chimie Thérapeutiques, 67000 Strasbourg, France
 E-mail: a.bianco@ibmc-cnrs.unistra.fr
²Dipartimento di Scienze Farmaceutiche, Università di Trieste, Trieste 34127, Italy
³University of London, Centre for Drug Delivery Research, The School of Pharmacy, Nanomedicine Laboratory, London, WC1N 1AX, UK