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Expert Review

Crucial Functionalizations of Carbon Nanotubes for Improved Drug Delivery: A Valuable Option?

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Abstract. Amidst the myriad of Drug Delivery Systems able to enhance delivery, absorption and intracellular uptake of a bioactive molecule while protecting it from deactivation, Carbon Nanotubes (CNTs) have emerged as a recent and promising option especially in cancer therapy. This is mainly due to their unique properties, which render them extremely versatile through the incorporation of several functional groups and targeting molecules at the same time, while their natural shape allows them to selectively penetrate across biological barriers in a non-invasive way. In this expert review we aim to evaluate whether this innovative material, once chemically-modified with suitable functionalizations, can be considered as a valuable system in comparison to the already existing nanodevices. This will include the estimation of the most recent advances in the field of nanotechnology, together with a cautious evaluation of potential risks and hazards associated with the extensive use of this fascinating, but still unknown, nanomaterial.

KEY WORDS: carbon nanotubes; drug delivery systems; functionalization; nanotechnology; toxicity.

INTRODUCTION

The intriguing combination of chemistry, physics and biology at the nanometric scale has been recently indicated as Nanotechnology, and it is also rapidly expanding into the biomedicine field. Small dimensions seem to be the key-component in the successful development of this multidisciplinary field, since they represent the ideal arrangement for a selective route in compartments within the cells and, at the same time, the possibility to escape from rapid entrapment and degradation in different organs and tissues. In particular, such powerful technology is predicted to have a large impact on life sciences and predominantly on cancer treatment (1–10). This breakthrough seems to be feasible due to the rapid development of nano-devices, which are small enough to extravasate through minute defects of the fenestrated vasculature, a characteristic phenomenon in tumor vessels (11). Among the myriad of nano-systems, derived from either simple or sophisticated materials, some of them have paved the way for a widespread use as drug delivery systems (DDS), based on their ability to transverse several physiological barriers, which still represent a challenging obstacle for drug targeting (12). For DDS it is important to consider the main limitations presented by several therapeutic agents such as poor solubility, rapid deactivation, unfavorable pharmacokinetic (PK) and limited biodistribution (BD). Not all the carriers—especially those for controlled release—can be interchanged, since their specific role is often based on different mechanisms; however, in general when a drug is incorporated in such a DDS, its elimination is reduced (with a concomitant increase in its half-life) and the volume of distribution decreases (13). Besides that, several DDS for drug targeting in cancer therapy take advantage from the unusual increased permeability of tissue vasculature (fenestration) that occurs in pathological conditions (the so called Enhanced Permeability and Retention (EPR) effect), thus enabling their bioactive molecule to be preferentially taken up in a localized area of the tissue. An example is given by the encapsulation of doxorubicin in pegylated liposomes, which were able to increase drug concentration in tumor tissue of about ten times in comparison to the free drug (14). Even though this "passive targeting" mechanism has been efficiently adopted by several delivery systems, many other devices take advantage of their intrinsic prolonged release (e.g. Depocyt, Cytarabine liposome injection) (15) or their natural

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ABBREVIATIONS: AmB, amphotericin B; BD, biodistribution; BNCT, boron capture neutron therapy; BSA, bovine serum albumin; CNTs, carbon nanotubes; DDS, drug delivery systems; DNA, deoxyribonucleic acid; DOX, doxorubicin; EDC, *N*-(3-dimethylaminopropyl)-*N'*-ethylcarbodiimide; EPO, erythropoietin; EPR, enhanced permeability and retention effect; FA, folic acid; *f*-CNTs, functionalized carbon nanotubes; FDA, Food and Drug Administration (USA); HIV, human immunodeficiency virus; HMM, altretamine or hexamethylmelamine; IR, infrared spectroscopy; MPS, mononuclear phagocyte system; MTX, methotrexate; MWCNTs, multi-walled carbon nanotubes; NHS, N-hydroxysuccinimide; NIR, near-infrared; NMR, nuclear magnetic resonance; NPs, nanoparticles; pCNTs, pristine carbon nanotubes; PEG, polyethylene glycol; PK, pharmacokinetic; RBM, radial breathing mode; RNA, ribonucleic acid; SDS, sodium dodecyl sulfate; STIs, sexually transmitted infections; SWCNTs, single-walled carbon nanotubes; TEM, transmission electron microscopy; UV, ultraviolet spectroscopy.

tendency of localizing to the mononuclear phagocyte system (MPS): for instance, the successful liposomal incorporation of Amphotericin B is used to treat leishmaniasis caused by a parasite resident within the macrophages (16). Finally, some other systems could be combined with surface antigens or receptors, thus giving origin to the well known "active or ligand-mediated targeting" (17).

In general, current DDS mainly include liposomes, dendrimers and polymers, virus-based systems, nanoparticles, fullerenes, nanohorns and nanotubes. Before their discovery, the efficacy of a therapy was somewhat empirical, based primarily on the physico-chemical properties of the drug itself in terms of size, dimensions and interaction with plasma binding proteins; on the contrary, the possibility of adopting a biocompatible platform able to protect the molecule of interest and to selectively target specific compartments without affecting the surrounding tissues, has induced several scientists to develop new systems. Therefore, with the purpose to avoid the unwanted side effects intrinsically associated with systemic administration, ideal drug delivery systems should liberate therapeutic agents to the target site without collateral adverse damage; indeed, such devices should aim to (a) protect a bioactive molecule from deactivation, (b) improve the pharmacokinetic profile of a drug and (c) enhance intracellular penetration and distribution (9). These systems seem mostly advantageous for those molecules that present good pharmacological profiles and whose application is nevertheless restricted by several problems such as low solubility or high toxicity towards normal cells. Due to their reduced size, usually ranging from 1 to 100 nm, these tools are particularly suitable for manipulations at the molecular level, and very often they have demonstrated to improve the pharmacological profile and therapeutic properties of the administered drugs, while limiting their toxicity.

In this manuscript we aim to consider whether the use of functionalized carbon nanotubes could represent a valuable alternative, taking into account advantages and drawbacks of currently available drug targeting vehicles,. We on purpose have limited our evaluation to the nanometer level without addressing larger scale systems and implants, in order to focus our attention on the real opportunities that nanotechnology in general, and nanomedicine in particular, might provide. Both nanomedicine and nanobiotechnolgy aim to a highly specific medical intervention at the molecular scale for diagnosis, prevention and treatment of diseases. Moreover, this expert review will discuss some recent examples of drug delivery from chemically-modified carbon nanotubes, which have shown promising results, and thus may positively influence the understanding, the cutting-edge research and the somehow "careful" application of carbon nanotubes as efficient drug delivery systems.

CURRENT DRUG DELIVERY SYSTEMS

Liposomes. As reported in Table I, there are several factors which either contribute or limit the biomedical applications of some drug delivery systems: unilamellar liposomes, for example, are vesicles made up of a lipid bilayer, similar to cells in terms of cell membrane, but with an empty nucleus. They represent the first generation of drug delivery systems and, due to their dimensions ranging between 90 and 150 nm, they are somewhat bigger than what we defined as Nanotechnology (≤100 nm). However, they can be modified in

Table I. Characteristics of Nanomaterials Used as Drug Delivery Systems

Nano-material	Size	Advantages	Disadvantages
Liposomes	100–200 nm	Excellent biocompatibility, low toxicity	Big dimensions Low transfection efficiency for gene delivery Possible physical instability in solution
Dendrimers and smart polymers	Variable, within nm	High controllable size and surface functionalization	Cytotoxicity (above 200 nM) Slow release rate (improved with pH-sensitive polymers)
Nano-particles	<100 nm	Controlled size and release Iron: hyperthermia, contrasting agents for MRI Gold: hyperthermia antiangiogenic and anti-inflammatory properties Viral: unique transfection efficiency, specificity	 Potential toxicity Toxicity to hepatocytes Quick mutation virulence
Fullerenes	1 nm	Easily functionalizable Resistance to biochemical degradation	Accumulation in liver (prolonged retention in the body) High binding to plasma proteins Potential toxicity
Nanohorns	80–100 nm	Large surface area No need of catalyst in the production Multiple drugs can be incorporated	Insoluble in aqueous media Self-assembling into agglomerates with potential toxicity

such a way that they are able to increase the solubility of amphiphilic drugs and prolong their circulation times for a sustained release of their contents (18,19). The main advantage relies on their excellent biocompatibility (through their easy integration inside cell walls after rupture), and the possibility to be converted into pH- or temperature-sensitive vehicles. The latest prototypes suggested by Needham's group combine drug-loaded-liposome with local hyperthermia, resulting in a complete regression of tumor growth in all human squamous cell carcinoma xenograft lines (FaDu) induced in the hind limb of athymic nude mice (20). Since their discovery in the early 1960s, several subtypes have been investigated and modified, finally leading to many formulations being already in phase II and III of clinical trials. Nevertheless, only a few liposome drugs (DaunoXome®, Doxil®, Caelyx®) have been approved by the Food and Drug Administration and are replacing conventional chemotherapy for the treatment of metastatic ovarian cancer. However, although showing excellent biocompatibility, liposomes as DDS may suffer from physical instability in solution due to their amphiphilic structure. In addition, doxorubicin-containing-liposomes (Doxil®) appear to contribute to superficial toxicity, usually referred as "hand and foot syndrome" (21,22). This effect seems to be associated with the prolonged circulation time of liposomes and the subsequent extravasation of the drug into the tumor tissue (23). Moreover, the inclusion of non-natural phospholipids for the liposome fabrication (especially those involving positive charges) might determine systemic adverse effects, most notably involving cellular components of blood and coagulation systems.

Dendrimers and "Smart" Polymers. Dendrimers are highly branched, multiple-shaped polymers, with a diameter's range within a few nanometers. Their main feature is their exquisite dimensional control and their vast exposed surface of profuse primary amines or multiple acids for an easy coupling with bioactive molecules. It is also possible to obtain multifunctional complexes, able to encapsulate a therapeutic agent, a targeting molecule and a fluorescent dye at the same time (24). Even if extremely promising in delivering molecules and nucleic acids, some poly(amidoamine) (PAMAM) dendrimer-based multifunctional conjugates have shown a significant reduction of cell viability (above a concentration of 200 nM) (24,25); it is still unclear whether the toxicity observed was due to the intracellular delivery of the drug candidate or to the delivery system itself. Hence further investigations are required to disclose the real process. In addition, the incorporation of anticancer drugs such as methotrexate and adriamycin inside the core of pegylated dendrimers determines a very slow

Therapeutic agents delivered	Applications	Status	Reference
Amphotericin B (Ambisome), doxorubicin (Doxil, Caelyx), doxorubicin + galactosamine daunorubicin, bleomycin, vincristine Paclitaxel Dexamethasone, thalidomide Irinotecan HCl, floxuridine Prostaglandine-E1…	Cancer therapy, autoimmune diseases	Phase II and III of clinical trials and drugs (DaunoXome®, Doxil®, Caelyx®)	(2,9,18–23,59) http://clinicaltrials.gov/ct2/results?term=Liposomes+and+Drugs
Paclitaxel Adriamycin Methotrexate Amphotericin B Microbicide (VivaGel)	Cancer therapy, fungal and microbial infection	Phase I	(2,24–26,59)
Mitoxantrone, doxorubicin Paclitaxel Cisplatin 5-fluorouracil (5-fluorouridine) Gemcitabine	Cancer therapy	Research Research	(9,31,32–41,59)
Gold sodium thiomalate	Rheumatoid arthritis	Drug (Ridaura®)	
Paclitaxel Vinblastine Docetaxel Streptavidin	Cancer therapy Pesticides	Phase II Market	(42–46)
Paclitaxel	Cancer therapy Antioxidant Antibacterial	Research Market (Zelens cream)	(47–54) http://www.nanotechproject.org/inventories/consumer/browse/products/5267/
Dexamethasone Cisplatin	Rheumatoid arthritis Cancer therapy	Research	(55–58,166)

release of less than 15% within 20 h. Therefore, with the aim of improving the delivery efficacy, some "smart" polymers have been recently developed; being sensitive to minimal changes in pH, they undergo endosomal disruption processes and subsequent endocytosis, thus increasing cytosolic delivery of the incorporated molecules and reducing drug toxicity. At the moment, the company Starpharma is focusing on the use of dendrimers as drugs by themselves, in contrast to dendrimers as drug delivery vehicles or diagnostics; in fact the dendrimer-based microbicide (VivaGel) has demonstrated to be effective in the prevention of HIV and sexually transmitted infections (STIs), after having completed successfully the first clinical trial (26). Another fascinating example regarding the direct use of nanodevices as a drug molecule has been provided by Ghadiri and co-workers (27): by alternating D- and L-amino acids, they prepared cyclic peptide nanotubes, whose diameter was finely controlled by the number of residues (28); these tools were used by Fernadanez-Lopez *et al.* (29) as a new class of antibiotics, on the basis of their ability to cross the cell walls of the bacterium, thus inducing a rapid cell death. Alternatively, they functioned as artificial ion channels in lipid bilayers by self-assembling transversely in the membrane, hence emulating the natural ion transport flux across the cell (30).

Nanoparticles. Besides organic nanoparticles such as liposomes and some dendrimerized material, inorganic nanomaterials including iron and gold nanoparticles, fullerenes, carbon nanohorns and carbon nanotubes seem to offer several advantages: they are usually easy to produce with a controlled size, they can incorporate multiple functions and therefore they might find application both as drug carriers, as well as targeting vehicles and contrast agents. On the basis of the general observation that synthetic materials larger than a few nanometers in size cannot penetrate cell membranes without modifying their integrity, nanoparticles of reduced diameters (below 100 nm) seem very promising: in particular the superparamagnetic iron oxide nanoparticles (31) offer the advantage to be easily modified to have either hydrophilic or hydrophobic character by simply coating them with a polyether backbone or aliphatic surfactants, respectively. Under the influence of an alternating magnetic field, they undergo Brownian relaxation, producing heat when moving in the field. Heat induced apoptosis to tumor cells is the basis of successful application of metal nanoparticles in cancer therapy. Recently, a new method of reversible association of doxorubicin (DOX) or mitoxanthrone and other antineoplastic drugs to superparamagnetic iron oxide nanoparticles has been developed to improve magnetically targeted chemotherapy (32–36).

Gold nanoparticles are apparently able to exert hyperthermic effects as well without being cytotoxic, and show the benefit to be precisely quantified till a density of 0.001 ppm by instrumental neutron activation analysis (37). In addition, unlike iron nanoparticles, they display antiangiogenic properties (through selective binding and inhibition of heparin-binding glycoproteins) and anti-inflammatory effects (through the reduction of antibodies and cytokine release) (38). As a confirmation of these properties intrinsically present in such material, gold sodium thiomalate (Auranofin or Ridaura®) has been successfully used and approved for the treatment of inflammation associated with rheumatoid arthritis (39,40). However, one of the key challenges in anticancer therapy is still the toxicity and poor targetability of the anticancer drugs. Patra and collaborators should have the credit by showing the pivotal role of gold nanoparticles in combination with Cetuximab as targeting agent and Gemcitabine as an anticancer drug in the reduction of the systemic drug toxicity, both in *in vitro* and *in vivo* studies (41).

Viral nanoparticles, in particular those incorporating adenoviruses, have demonstrated to be excellent matrices for gene therapy, vaccines and drug delivery, on the basis of their high transfection efficiency and specific receptor-binding properties (42). In initial studies, however, responses were generally transient and limited to the site of injection, because the natural immune system is responsible for the clearance of viral particles, thus attenuating their potency. For that reason, the combination therapy with additional drug molecules has been investigated for an improvement of the viral activity. For example, Paclitaxel stabilizes microtubules during M phase and increases adenoviral binding and transgene expression (43,44). As a result, agents such as Paclitaxel, Vinblastine, and Docetaxel seem to contribute to increased efficacy when combined with adenoviral therapies. In addition, enhanced uptake of Streptavidin (a protein with clinical applications in anticancer therapies) was demonstrated for protein–carbon nanotubes conjugates without affecting cell viability or toxicity (45), suggesting that the hybrid virus-nanotube network could enhance the molecule's activity. At the same time, a deeper understanding of viral biology has led to the modification of the viral genome in order to better address therapeutic issues: recombinant viruses have already found application in agricultural biotechnology as potent pesticides (46). Unfortunately, these viral devices might undergo quick mutations which can lead unspecific toxicity and undesirable effects upon delivery. This is attributable to the fact that their potency seems to be directly correlated with their virulence towards human tissue, thus inducing deep uncertainty in terms of safety and protection.

Fullerenes and Nanohorns. Paclitaxel was also delivered in the form of a slow release formulation (47) from fullerenes, an allotropic form of carbon discovered in the middle '80s and presenting a buckyball structure with a diameter of about 1 nm. This unique material has been extensively used (48) for its ability as antioxidant (49,50), antibacterial (especially when bearing positive charges) (51), contrast agent (52,53) and sensitizer for photodynamic therapy (54). The big limitation is that fullerenes are retained in the organism for prolonged periods, since they tend to bind to plasma proteins and accumulate mainly in the liver, thus prohibiting any biomedical applications until chronic toxicity will be completely disclosed.

Recently, carbon nanohorns have been investigated for their ability to incorporate a drug in their interior space, while being conjugated with another one on the external walls, which consist of closed single walled carbon nanotubes. In comparison to this last-mentioned material, nanohorns offer the great benefit to be better controllable in their holes shape (55) and produced without the need of any catalyst that often remains as a toxic residual in several kinds of nanomaterials. Therefore, they seem particularly promising in the development of multi-drug therapies: an interesting result has been obtained by Iijima and collaborators, who were able to entrap and then release the anti-inflammatory drug dexamethasone (56) and the anticancer agent cisplatin (57). In both cases, the

samples exhibited a sustained release of the biologically active molecules with rates that were affected by the hole-edge structure (that is either with or without oxygen-containing functional groups). At present, a significant drawback to their future advance is represented by their tendency to self-aggregate into agglomerates (58) that, being in the micrometer scale (superior than 4 μm), might result in vascular occlusion or localized toxicity.

Many other tools are currently available for therapeutic release and also for imaging (e.g. nanoshells and quantum dots) or sensing (e.g. nanowires) (59), but they are outside the scope of this review. Anyway, it is worth mentioning that at the moment scientists are deeply investigating the combination of more than one vehicle in order to build versatile platforms able to specifically target, efficiently deliver and proficiently visualize the site of actions of these multifunctional conjugates.

FUNCTIONALIZED CARBON NANOTUBES AS DRUG DELIVERY SYSTEMS

Nanosized DDS represent one of the most interesting results deriving from the development of advanced materials for biomedical and biotechnological applications (60). Among the numerous delivery systems currently under investigations, carbon nanotubes (CNTs) seem to embody a promising option (61). Pristine carbon nanotubes (pCNTs) are made up of carbon atoms arranged in a series of condensed benzene rings and wrapped into a tubular form (Fig. 1). Regardless whether they contain either one (SWCNTs) or multiple (MWCNTs) graphene sheets, they present several interesting properties, such as high aspect-ratio, ultra-light weight, tremendous strength (62), high thermal conductivity (63) and remarkable electronic properties ranging from metallic to semiconducting (64–66). It is not clear yet which of the two systems is more advantageous: SWCNTs offer the additional photoluminescence property that could be proficiently applied in diagnostics, while MWCNTs present a wider surface that allows a more efficient internal encapsulation and external functionalization with active molecules (67). They have both been used for diversified roles including biosensors (68), field-effect transistors (FET) (69,70), and scanning probe elements (71).

Concerning their use in biological systems, lack of solubility (both in organic solvents and aqueous solutions), formation of thick and inhomogeneous bundles, circulation half-life of 3–3.5 h (72), biocompatibility and immunogenicity limitations provide sufficient evidence to arise great concerns. However, these observations hold only for pristine pCNTs and therefore just indicate the need for further modifications in order to explore the feasibility of functionalized CNTs (*f*-CNTs) as safe bio-nano-material. Conversely, the advantage of incorporating multiple functions at their surface, the ability to render them dispersible in aqueous media (73) and the possibility to use them as scaffolds for cells growth (74) have stimulated the curiosity of several scientists.

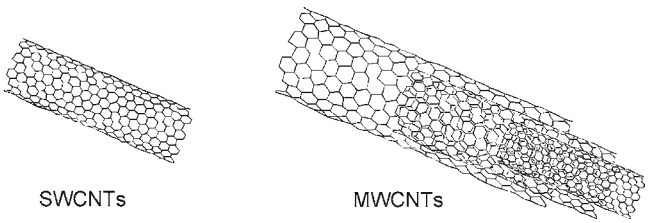


Fig. 1. Single-walled (on the *left*) and multi-walled (on the *right*) carbon nanotubes.

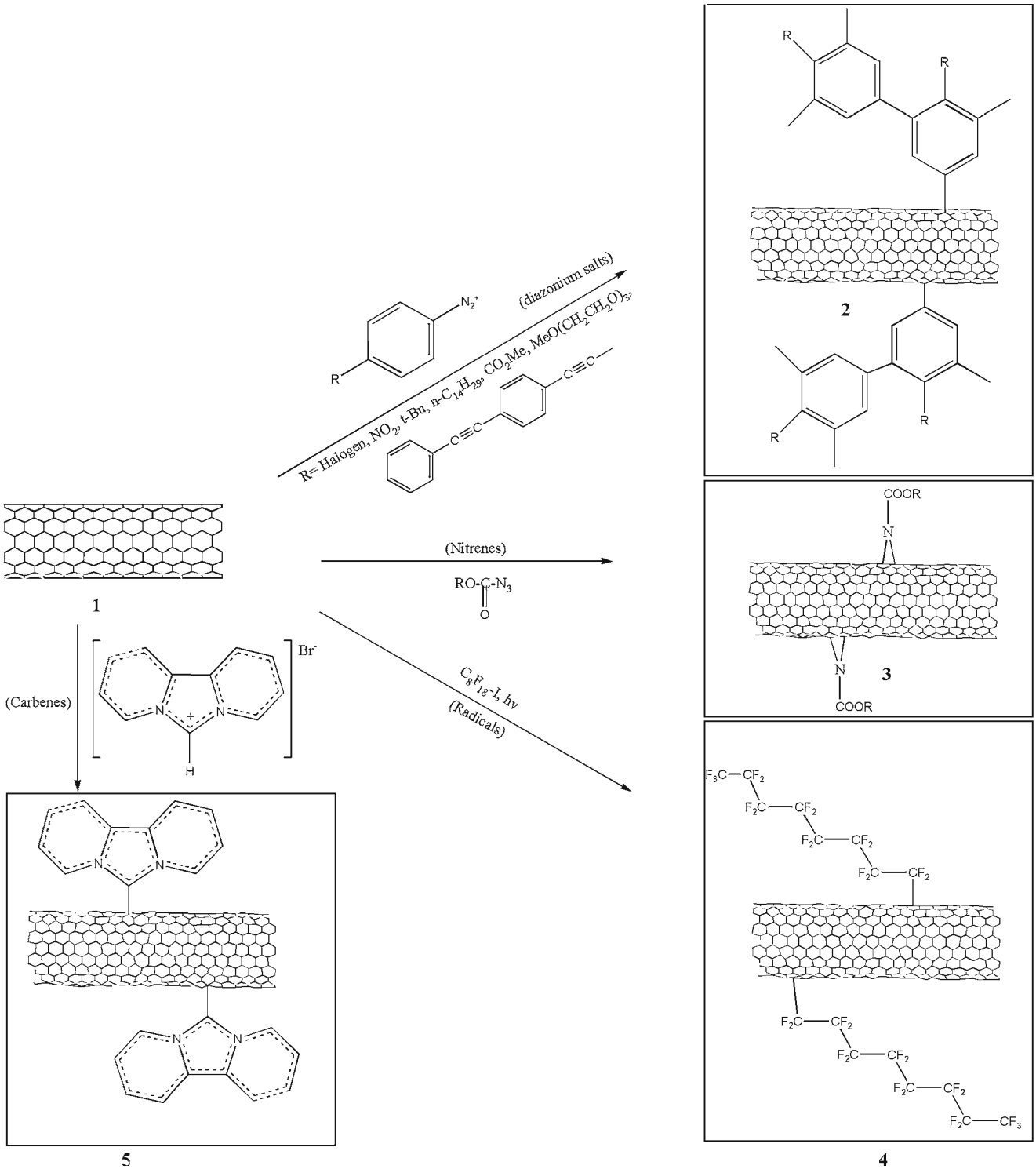
In particular, the application of *f*-CNTs as new nanovectors for drug delivery became doable soon after the demonstration of cellular uptake of this new material (45,61,75–78). It is worth to mention that, apart from a few cases of phagocytic incorporation inside macrophages (79,80) (which are known to be large cleaning cells able to remove stranger material including less soluble nanotubes), no naked pCNTs were reported to penetrate inside cells without displaying remarkable effect. This last point should reinforce the use of *f*-CNTs as improved, less harmful nanovehicles, especially after our recent discovery regarding the lack of a direct correlation between the kind of functionalization on the surface of carbon nanotubes and their internalization extent (81): either electrostatically neutral or charged *f*-CNTs could be taken up by cells with comparable amount, hence indicating that numerous, different chemical procedures could be adopted to introduce several groups and functionalities.

Since derivatization of the tubes is recommended to improve their processibility, scientists have deeply exploited the chemical properties of carbon nanotubes for example (1) through the supramolecular complexation with detergents (82–84) and polymers (85), (2) through the further conversion of carboxylic functionalities introduced mainly at the tips by oxidative conditions (86–88) or (3) through direct addition reactions (89) to the unsaturated π-electron system of the nanotubes' sidewalls via carbenes, nitrenes, radicals or diazonium salts (90–97) (Scheme 1).

However, even though a lot of diversified CNTs' functionalizations have been successfully achieved, only few examples of delivery of small molecules (antibacterial, antiviral and anticancer agents) using *f*-CNTs are currently reported in literature (Table II). This could be attributed to the fact that even the multiple functionalization of nanotubes utilizes only a limited portion of the whole surface available, and thus the drug loading does not allow to guarantee the desired pharmacological effect; in addition, no application for drug delivery has been approved or entered the market yet, favoring an increasing skepticism toward any bio-application of these nanotubes. Nevertheless, although at the moment liposomes are still much more promising and less problematic than CNTs in terms of drug delivery, it is also important to realize that there are at least 30 years of difference between the discovery of these two devices. Many interesting results may be expected in the very next future, showing that CNTs should be much deeper investigated for their potential impact in nanoscience.

Non Covalent Functionalization on the External Walls

At the moment, functionalization of carbon nanotubes for their application in the biomedical field is mainly restricted to those chemical strategies able to render this material biocompatible as well as functional: the simplest procedures include the physical adsorption of pCNTs to several molecules such as pyrene, naphthalene derivatives (98), sulfonated polyaniline (99), poly(acrylic acid) (100), proteins and DNA (101–103) and gold nanoparticles (104). The nanotube-adsorbate conjugation is caused by π–π stack-



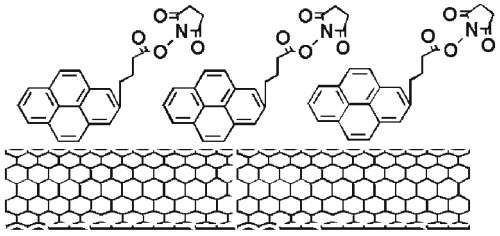
Scheme 1. Surface functionalization of CNTs via (in a *clockwise order*): diazonium salts, nitrenes, radicals and carbenes.

ing interactions between the aromatic part of the adsorbate and the graphitic sidewall of nanotubes, without affecting CNTs' whole integrity. An interesting case is given by the *N*-succinimidyl-1-pyrenebutanoate (Scheme 2), which on one side enabled the irreversible adsorption of the pyrenebutanoate part onto the surface of SWCNTs through π–π interactions, while its succinimidyl ester group allowed the covalent attachment of various molecules via the nucleophilic attack of primary or secondary amines (such as in ferritin, streptavidin or biotin-polyethyleneoxyde-amine) (82).

The remarkable increase in CNTs aqueous dispersibility (85,105–108) is another beneficial effect of this interaction, and it has been adopted to purify CNTs from contaminations represented mainly by amorphous carbon (109–111); in this

Table II. Functionalizations of Carbon Nanotubes (CNTs) and Their Use as Drug Delivery Systems (DDS)

	CNTs	Diameter	Functionalization	Therapeutic agents incorporated	Applications	Reference
Non covalent physical adsorption on CNTs' external walls	pSWCNTs	~1-nm	1-pyrenebutanoic acid, succinimidyl ester onto CNTs	Proteins (ferritin & streptavidin)	Cancer therapy	(82)
			Amphiphilic (PEG)-based copolymer	Doxorubicin (DOX)	Cancer therapy	(119)
			Co-polymer Pluronic F127	Doxorubicin (DOX)	Cancer therapy	(121)
			PEG-8 caprylic/capric glycerides	Erythropoietin (EPO)	Anaemia	(122)
			Phospholipid (PL)–folic acid (FA) copolymer + NIR	–	Cancer therapy	(123)
			–	DNA	Gene therapy	(144)
			PEG-platinum(IV) construct	Prodrug Pt(IV)	Cancer therapy	(172)
	SWCNTs-ox	~1-nm, shorter tubes	SWCNTs-CONH-$C_6H_{12}NH_3^+$	siRNA	Cancer therapy	(171)
	pMWCNTs	10–50 nm	Co-polymer Pluronic F127	Doxorubicin (DOX)	Cancer therapy	(121)
Covalent functionalization	SWCNTs-ox	~1-nm, shorter tubes	Oxidation + EDC + Biotin/Streptavidin	Streptavidin	Cancer therapy	(45)
			Oxidation + carbodiimide (EDAC)	BSA	Immunohistochemistry, enzyme stabilization	(131)
			Oxidation + EDC	DNA	Gene therapy	(132,133)
	MWCNTs-ox	10–50, shorter tubes	Oxidation + carbodiimide (EDAC)	BSA	Immunohistochemistry, enzyme stabilization	(131)
			Oxidation + EDC + NHS	Gonadotropin releasing hormone	Cancer therapy	(173)
	SWCNTs	~1-nm	Nitrene cycloaddition_substituted C_2B_9 carborane units	Boron	Boron capture neutron therapy (BNCT)	(92)
	MWCNTs	10–50 nm	1,3-dipolar cycloaddition of azomethine ylides	Methotrexate (MTX)	Cancer therapy	(135)
			Oxidation and 1,3-dipolar cycloaddition of azomethine ylides	Amphotericin B (AmB)	Fungal infection	(137)
Encapsulation	SWCNTs	~1-nm	Annealing at 350°	β-carotene	Photonic technology	(148)
	SWCNTs + DWCNTs	~2-nm	Annealing at 550° + nano-extraction	Altretamine (HMM)	Cancer therapy	(160)
	MWCNTs	10–50 nm	Thermal treatment + oxidation	Carboplatin	Cancer therapy	(164)



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Scheme 2. *N*-succinimidyl-1-pyrenebutanoate physically adsorbed on the surface of SWCNTs through π–π interactions.

way, impurities are likely to sediment, thus remaining separated from freshly suspended nanotubes.

With similar methodologies, tubes' unboundling can be easily obtained through the use of surfactants (82,84,112–116) or solubilizing polymers (85,117). In this case, hydrophobic interactions play a determinant role: several surfactants (either anionic, nonionic or cationic) are able not only to suspend carbon nanotubes in aqueous solution (112–115), but also to prevent re-aggregation of the tubes by coulombic repulsion between surfactant coated CNTs (117). Moreover, if the amphiphilic molecule presents aromatic groups in its hydrophobic part, additional π–π stacking interactions take place with the graphitic sidewalls of CNTs, while hydrophilic groups are exposed to the aqueous solution. Some of the surfactants such as sodium dodecyl sulfate (SDS) have resulted to be very effective in dispersing individual SWCNTs, opening new opportunities to study and to further manipulate single tubes (118). Similarly, water-soluble polymers like polyvinylpyrrolidone (PVP) and polystyrenesulphonate (PSS), uniformly wrapped around the surface of SWCNTs, facilitated nanotubes' dissolution in aqueous phases (117). The advantage is that the association of SWCNTs and polymer is largely due to thermodynamic forces that eliminate the hydrophobic interface between tubes and aqueous medium. This phenomenon is reversible by simply changing the solvent system, thus opening the way to more accurate manipulation, purification and functionalization of this material.

Even though water-dispersibility of CNTs is extremely important and re-aggregation of the tubes should be minimized, the above-mentioned procedures do not always allow an effective, stable incorporation of additional bioactive molecules. Therefore SWCNTs cannot be classified as efficient DDS. An exception in this sense is represented by the recent work of Park and collaborators (119), who designed a "trivalent" amphiphilic polymer: in other words, they first prepared a polymer bearing both hydrophobic and hydrophilic residues and thus able to disperse CNTs in water; second, they integrated a polyethylene glycol (PEG)-based copolymer useful as solubilizing agent and as protection against protein adsorption (the so-called anti-biofouling effect, which is often encountered during *in vivo* studies). Finally, they attached an anticancer drug, doxorubicin (DOX), whose positively charged amino groups interacted with the carboxylic functions of the polymer, while its aromatic rings stabilized the π–π stacking interactions at the surface of CNTs. Non-covalent functionalization with polyethylene glycol (120) or block co-polymer Pluronic F127 (121) was also used to adsorb the same drug (DOX) onto SWCNTs and MWCNTs, respectively. Noticeable different results might imply that further mechanistic evaluation should disclose whether the cell internalization of CNTs is also affected by drug/nanotube complexes. With a similar procedure, Ito and co-workers discovered that short fibers of CNTs were able to increase both delivery and absorption of erythropoietin (EPO), identifying key factors to improve oral delivery of drug proteins (122).

Another extraordinary insight of *f*-CNTs in cancer therapy is represented by the recent work performed by Kam and colleagues (123): after the interesting observation that biological systems are transparent to 700- to 1,100-nm near-infrared (NIR) light, the intrinsically strong optical adsorbance of SWCNTs within the same range was exploited to achieve selective cancer cell destruction. In other words, pristine SWCNTs wrapped with a PEG moiety modified with a phospholipid (PL) chain on one side and folic acid (FA) on the other side, not only permitted the selective internalization of the complex inside tumor cells (that usually overexpress folate receptors (FRs) at their surface and facilitate cellular uptake of folate-containing species by ligand-mediated endocytosis), but also determined a specific cancerous cell death, while preserving healthy cells after laser radiation at 808 nm. The advantage of this technique lies on the ability of laser pulses to induce local heating and death only of those tumor cells that had internalized the *f*-CNTs, thus paving the way for exciting new developments for cancer therapy by sophisticated DDS.

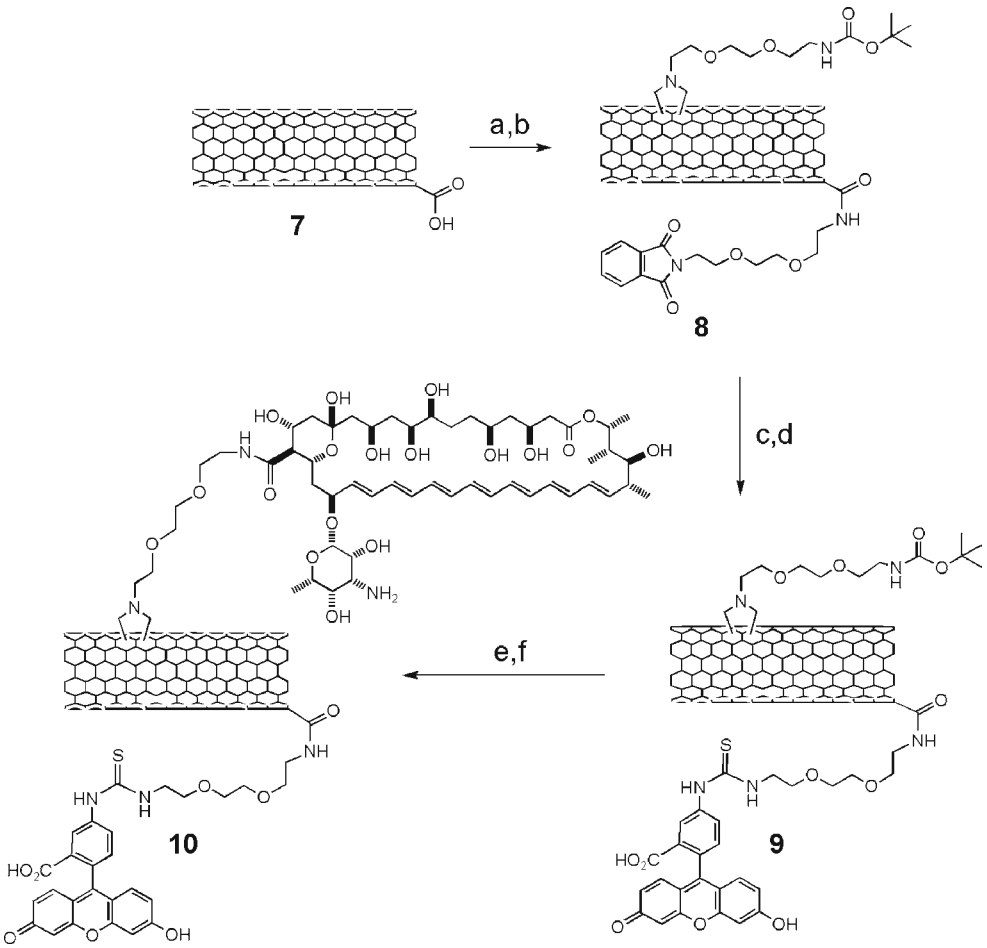
"Defect" Functionalization at the Tips and Sidewalls

Besides non-covalent procedures, CNTs can also be cut and functionalized simultaneously, becoming soluble in polar organic solvents, acids and water without the aid of sonication, surfactants, or any other means, by simply treating them with oxidizing agents such as HNO_3, $KMnO_4/H_2SO_4$, O_2, $K_2Cr_2O_7/H_2SO_4$ or OsO_4 (82–84). After that, it is possible to use the carboxylic acid groups and the carboxylated fractions (124) introduced by oxidization treatment, to further functionalize the nanotubes via amidation, esterification or through the zwitterionic $COO^-NH_3^+$ formation. This often requires activation of the carboxylic acids by thionyl chloride, N-hydroxysuccinimide (NHS), or carbodiimide (e.g., *N*-(3-dimethylaminopropyl)-*N*′-ethylcarbodiimide, (EDC); *N*,*N*′-dicyclohexylcarbodiimide, (DCC)) in order to get highly reactive intermediates. Subsequently, various lipophilic and hydrophilic dendrons could be attached to CNTs via amide or ester linkages, and they offer the advantage of improving the solubility of CNTs in organic or aqueous solvents (125), as well as being easily removable under basic or acidic hydrolysis conditions (126). This oxidizing procedure is usually known as "defect functionalization", since it takes place at the ends or in correspondence of pre-existing defects of CNTs; if done under mild conditions it preserves the macroscopic features of CNTs without losing their electronic and mechanical properties (127–129), and it has being used to graft polymers like PEI-EI (poly-propionyl-ethylenimine-co-ethylenimine), PVK-PS (poly-*n*-vinylcarbazole) and PEG to the surface of CNTs (130). In addition, it has been employed to link biological molecules to CNTs via stable covalent bonds. For example, Huang and collaborators incorporated bovine serum albumine (BSA) on *f*-CNTs (both single and multi-walled) via diimide-activated

amidation, demonstrating that the protein, once bound to nanotubes, remained active (131). Analogously, streptavidin (a protein with potential clinical applications in anticancer therapy) was complexed to SWCNTs prefunctionalized with biotin through EDC activated amidation (45). DNA was also bound to CNTs via amide linkage (132,133) and reversibly hybridized with its complementary sequence, offering the possibility to reutilize the derived single-strand DNA-CNTs in a second-round of hybridization. To summarize, this process introduces carboxylic groups and carboxylated fractions that enable further manipulation and investigation of both the activity of incorporated biomolecules and the spectroscopic properties of *f*-CNTs. However, it can also introduce an excess of defects or determine ultra-short *f*-CNTs (134).

Covalent Functionalization on the External Sidewalls

Among the most powerful methodologies aimed to functionalize CNTs, a special kind of 1,3-dipolar cycloaddition represents a fascinating example of covalent bonding: it is extremely versatile, since it requires only an α-amino acid (or correspondent ester) reacting with an aldehyde or keton, to generate *in situ* azomethine ylides that are very reactive and thus determine the formation of pyrrolidine rings on the sidewall of CNTs (Scheme 3). The amount of pyrrolidine groups can be estimated via electronic absorption spectroscopy or calorimetric analysis, while the *f*-CNTs can be evaluated by standard spectroscopic techniques: even though the signals are broadened, the information obtained from NMR, UV and IR analyses could easily confirm that functionalization was achieved correctly. In general, the covalent functionalization of nanotubes is more robust and better controllable compared to procedures based on non-covalent methods, and it offers the possibility of introducing multiple functionalities. This last point seems particularly important in providing justification for the application of *f*-CNTs as DDS: first of all, the molecular targeting of CNTs carrying a bioactive molecule can be effective if an active recognition derivative is simultaneously expressed at their surface. Second, incorporation of a fluorescent dye would provide optical signal for imaging and localization of the eventual CNTs-drug conjugate. Therefore, our group has developed some strategies to integrate multiple groups on the tubes' sidewalls. One of them consists on the application of the 1,3-dipolar cycloaddition of azomethine ylides, to introduce N-functionalized pyrrolidine rings on the external walls of the tubes. Formaldehyde was added to two "orthogonally" protected α-amino acids, meaning that the removal conditions of one group did not interfere with those of



Scheme 3. *a* Neat $(COCl)_2$; Pht-$N(CH_2CH_2O)_2$-CH_2CH_2-NH_2 in dry THF at reflux. *b* Boc-$NH(CH_2CH_2O)_2$-CH_2CH_2-$NHCH_2COOH/(CH_2O)_n$ in DMF, 125°C. *c* Hydrated NH_2–NH_2 in EtOH at reflux. *d* FITC in DMF. *e* HCl 4M in dioxane. *f* Fmoc-AmB, HOBt/EDC×HCl/DIEA in DMF; 25% piperidine in DMF.

the second amino acid; in our specific case, the selective deprotection of the phthalimidic group (Phth) in ethanolic hydrazine allowed the introduction of the fluorescent molecule (FITC), while the acidic environment removed the *tert*-butyloxycarbonyl (Boc) group and successfully coupled the so-generated free amino function with the activated α or γ carboxylic group of the anticancer drug methotrexate (MTX) (135). Methotrexate is a drug widely used against cancer, but it displays toxic side effects and a reduced cellular uptake (136). Therefore, in this study we tried to overcome the limited capacity of MTX to cross the cell membrane by conjugating it to *f*-CNTs, which are capable to enhance cell uptake of linked moieties. Epifluorescence and confocal analysis of human Jurkat T lymphocytes, incubated with different concentrations (between 0.05 and 5 μg mL^{-1}) of these samples confirmed the presence of fluorescent tubes inside the cells around the nuclear membrane and clearly showed time and dose dependence of the internalization process.

In a slightly different approach, we have functionalized MWCNTs with amphotericin B (AmB), which is a potent antimycotic drug normally used for the treatment of chronic fungal infections (137). However, it also displays a remarkable toxicity towards mammalian cells (138), presumably because of its low water solubility and its tendency to form aggregates (139). Multi-walled CNTs were treated under strong acidic conditions for 8 h to reduce their length to about 180–940 nm. The carboxylic groups were coupled with a phthalimide mono-protected triethylene glycol diamine. Subsequently, the tubes underwent the 1,3-dipolar cycloaddition reaction to introduce N-functionalized pyrrolidine rings, bearing Boc-protected amino groups, on the external walls of the tubes (Scheme 3). Being the two protecting groups reciprocally orthogonal, they allowed the incorporation of a fluorescent dye to follow the cell internalization of the conjugate, while the drug molecule AmB could exert its antifungal action. In this study it was demonstrated that free-AmB at a dose of 10 μg mL^{-1}, corresponding to the amount of drug covalently bound to 40 μg mL^{-1} of MWCNTs, determined more than 40% of cell death, while all cells remained alive following the treatment with AmB-CNTs conjugate. Very interestingly, AmB preserved its high antifungal activity once linked to the nanotubes: to verify that, different types of pathogens comprising *Candida albicans*, *Candida paropsilosis* and *Cryptococcus neoformans* were treated with AmB-CNTs conjugates and the results were in some cases superior than those for the drug alone. Although the reason for such improvement in the activity is still unclear, it might be that the increase in the solubility of the drug, together with its favorable multipresentation to fungal membrane by CNTs, determined the enhancement of its therapeutic effect by decreasing mammalian toxicity and increasing the antifungal activity.

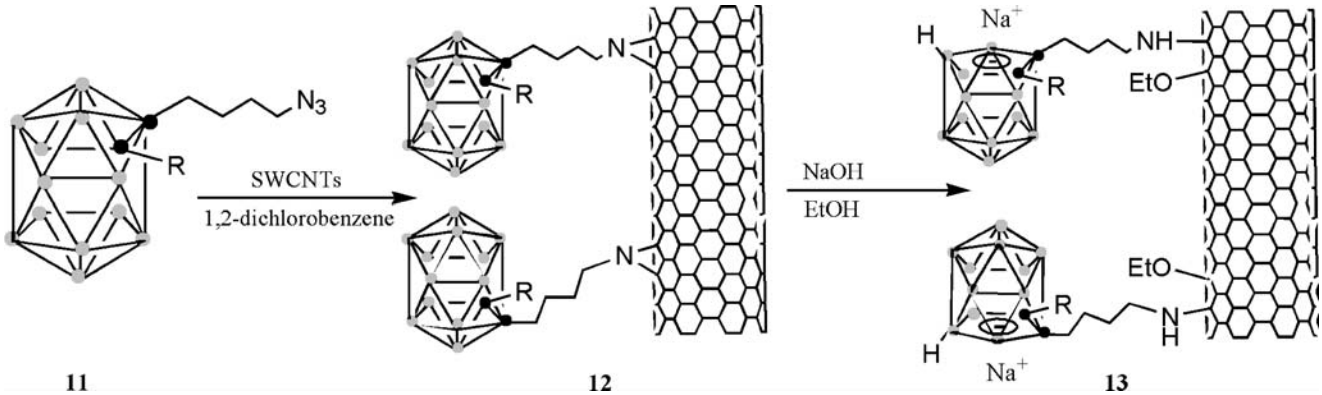
In addition, it was observed that AmB-CNTs conjugates were rapidly internalized into Jurkat cells in a dose-dependent manner and with a mechanism that excluded endocytosis, since the incubation of the cells at 4°C or in the presence of sodium azide (NaN_3, a well known inhibitor of energy-mediated processes) did not completely block the uptake.

There is still much of discrepancy regarding the uptake mechanism, namely, nanopenetration by insertion/diffusion as experimentally demonstrated mainly by Pantarotto and collaborators (75,76,137,139–142) contrary to phagocytosis/endocytosis internalization processes as proposed by other groups (45,79,143–145). The great divergence seems to be attributable to significant differences both in the nanotube materials and in the experimental procedures: for example, Dai's group suggested a specific clathrin-dependent mechanism as the principal pathway for intracellular transport of a) oxidized SWCNTs non-covalently conjugated with proteins (either bovine serum albumin (BSA) or streptavidin (SA)) and b) pristine pSWCNTs complexed with DNA molecules (144). In both cases, the non-covalent interaction was sufficiently strong to allow the entry as carrier-molecule complex into mammalian cells, as demonstrated by the impressive images under confocal fluorescence microscopy. A confirmation of the successful uptake derived from the weak fluorescence observed for the same proteins and DNA molecules but without nanotubes transporters. In addition, the low fluorescence levels after incubation at 4°C and in the presence of NaN_3, together with the disruption of clathrin-coated vesicles by pretreatment of cells with either sucrose or a K^+-depleted medium, clearly suggested a clathrin-dependent endocytosis at the basis of the internalization process. Interestingly, poor or non-existent cellular uptake was observed for large proteins (e.g. human immunoglobulin), presumably due to the large size of the cargo or to inefficient endocytosis of big conjugates (143).

Conversely, in order to evaluate the suitability of CNTs as transporters of large and heavy groups into the cells without toxicity, Hosmane and co-workers incorporated substituted C_2B_9 carborane units onto the side walls of SWCNTs via nitrene cycloaddition (92) (Scheme 4). They subsequently investigated the applicability of these *f*-CNTs in boron capture neutron therapy (BNCT) through a biodistribution study on different tissues. Results showed that, following a treatment with sodium hydroxide, able to render carborane-nanotubes water-soluble, these conjugates were likely to concentrate more in tumor cells than in blood, liver, lung or spleen when administered intravenously in mice. It is worth to note that unbound borane and carborane anions did not show preferential absorption or retention in tumor cells, thus reinforcing the importance of *f*-CNTs as boron delivery systems. Even though preliminary findings were particularly promising, further investigations are needed to provide more details on the mechanism involved and on cytotoxicity displayed before an extensive use of these ensembles for BNCT treatment of cancer. On the whole, the unambiguous diversity regarding the starting materials and the numerous kinds of functionalization (covalent *versus* non-covalent) employed by different research groups do not allow an exhaustive conclusion on the preferential pathway of this nanomaterial; therefore we should not exclude any other mechanism of internalization until further experiments will disclose new insights on this important issue.

Encapsulation Inside CNTs

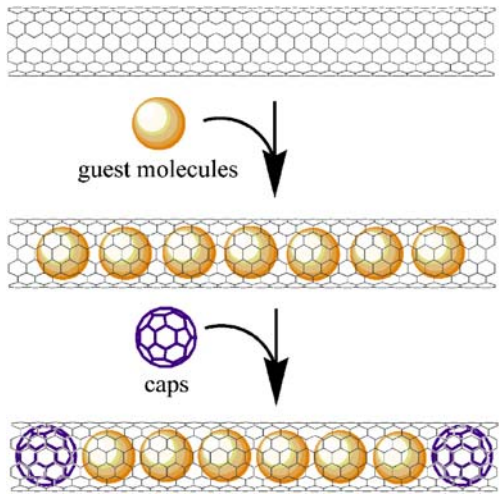
Although many biomolecules, adsorbed or bound onto the surface of nanodevices, have been mentioned to display an improved therapeutic activity, i.e. an increased water dispersibility, a better bioavailability and a reduced toxicological profile, there are many other examples showing that the interaction with the carrier or the surrounding environment could determine



Scheme 4. Synthesis of carborane substituted SWCNTs. *R* = Me or Ph; *gray circle* = BH; *black circle* = C.

inactivation or even degradation of these molecules. For that reason, the recent use of CNTs to encapsulate molecules has rendered these nanosystems particularly suitable for additional applications such as material storage (146), compound synthesis (147) and drug delivery (148). The successful encapsulation of organic molecules inside SWCNTs has already been reported (149–154); the advantage of this methodology lies on the ability of carbon nanotubes to provide protection and to control the release of loaded molecules, thus prolonging the effect of eventual drugs. An interesting example is the incorporation inside CNTs of a natural pigment, β-carotene (148), whose application as photonic tool substance has been hampered by its fast degradation, under light exposure, due to isomerization or reaction with radicals (e.g. singlet oxygen) (155,156). In this study, SWCNTs were initially opened by annealing at 350°C for 20 min; the subsequent encapsulation of β-carotene was confirmed by Raman analysis; this technique showed 3 characteristic peaks associated with carbon nanotubes, namely D band (at 1,250–1,450 cm^{-1}), G band (at 1,550–1,600 cm^{-1}) and radial breathing mode (RBM, below 350 cm^{-1}). Inclusion of fullerene (157) or other material usually results in the shift of the RBM peaks, while D and G bands are more affected by binding and adsorption of molecules on the external walls of CNTs, and therefore provide direct indication of the extent of sidewall functionalization (158,159). From the results obtained in these experiments, the crucial role of carbon nanotubes was obvious in the protection of the natural substance from an easy degradation, but no details were provided about its delivery.

For that reason, our group has investigated the possibility to incorporate a bioactive molecule inside carbon nanotubes with the purpose to provide protection, storage and controlled release (160). In our case, we adopted one of the procedures introduced by Iijima's group to encapsulate fullerene particles and defined as "nano-extraction" (161): for this process to happen, the mutual interactions among graphene sheets, molecules and solvent must be accurately balanced, in the sense that both CNTs and guest molecules must have poor affinity to the solvent, but strong reciprocal attraction. If these conditions are ensured, the desired molecule can be deposited within the CNTs as the most stable site. Taking into account such requirements, we initially heated CNTs at 550°C to open the tips of the tubes, and then performed a two-step nano-extraction to initially load an anticancer drug, hexamethylmelamine (HMM), inside SWCNTs and subsequently to seal the tubes with fullerenes (C_{60}) (Scheme 5). In this way, it was possible to obtain fascinating "nano-bottles" able to store and protect the guest molecules, as confirmed by TEM images and by extensive analyses. Even though the picture quality was disturbed by the unstable movement of isolated tubes, it was possible to recognize the tips of the tubes filled with C_{60} while the central part, presumably occupied by HMM, appeared empty. Differently from fullerenes, HMM's structure is not clearly evident under TEM, but its presence was confirmed by characteristic peaks in the RBM band of Raman analysis, which were not present in the control (CNTs filled just with C_{60}) and in complete agreement with previous studies reporting analogous shift of RBM after molecule loading inside the tubes (162,163). A further development consisted in the demonstration that it was possible to open these nano-bottles and to extract the entrapped drug: for that purpose, we used CH_2Cl_2 as solvent for its ability to readily dissolve both C_{60} and HMM. IR analysis resulted very useful to confirm the effective release; CNTs usually present a broad signal over the whole range from 400 to 4,000 cm^{-1}, but diluted solutions, aimed to minimize the interference from CNTs, allowed the detection of particular peaks at 2,900 cm^{-1} in the HMM-loaded-CNTs and their



Scheme 5. A "carbon nano-bottle" loaded with guest molecules and C_{60} using a controlled nano-extraction strategy. C_{60} filled at the extremities of CNTs can act as "caps" to seal the CNTs.

disappearance once the drug was released. The absence of these peaks, reasonably attributable to HMM's methyl groups, implied the successful removal of C_{60} and the extraction of the guest molecule, hence suggesting that CNTs could be further functionalized at their sidewalls for an improved targeting while protecting the encapsulated molecules.

Another interesting encapsulation of a drug inside CNTs has been obtained by Hampel's group, who also investigated the influence of CNTs on tumor cell growth (164). The additional value of their manuscript relies on two main aspects: first, the incorporation of carboplatin, a more water soluble and less neuro- and oto-toxic drug than its parent derivative cisplatin, is easily visible under TEM; second, CNTs presenting a wider inner diameter (MWCNTs) were used, hence they determined a higher drug loading that was further increased by controlled heat application. CNTs were opened by both thermal treatment and strong acidic conditions, while the drug was incorporated through a wet chemical approach, in which capillarity acted as the driving force. This last characteristic is an intrinsic property of opened CNTs and it is directly proportional to the energies of interaction between the solid surface of CNTs and the liquid. Ebbesen (165) established a cut-off of 100–200 m*N*/m as the surface tension value of liquids below which CNTs attract substances inside by capillary forces. Water, showing a relatively low surface tension of 72 m*N*/m, was thus able to fill MWCNTs with an optimum of 30% of carboplatin at 90°C. The subsequent cell-viability assays revealed that treatment with free carboplatin resulted in a concentration-dependent decrease of cell number and an increase in cell apoptosis, with about 50% of cells alive at a concentration of just 20 μg/ml. Interestingly, empty CNTs did not affect cell-viability; on the contrary, the addition of carboplatin to empty tubes determined a synergistic effect, probably because MWCNTs altered the integrity of the cell membrane and increased the uptake of the drug. These results suggested that, even though the long-term influence of CNTs on cells should be deeply investigated, carbon nanotubes seem to be promising carriers with remarkable mechanical and chemical stability, although with still unclear immunogenic effects.

PERSPECTIVES, CHALLENGES AND SKEPTICISM OF CNTS FOR CANCER THERAPY

The rapidly advancing area of cancer nanotechnology has generated many efforts in order to find potent drugs, selective targeting and efficient delivery; even though excellent findings have been shown recently, the success of numerous therapies is often hampered by several limitations, including resistance due to physiological barriers, low biodistribution and extensive clearance of anticancer drugs. The reason at the basis of a reduced efficacy is that many anticancer molecules present physicochemical properties that are not suitable for the diseased area, and thus require high doses, with concomitant toxicity and unwanted side effects. Therefore, new tools are required to overcome these inadequacies, possibly able to target tumor tissues and to deliver a cytotoxic warhead exactly where its presence and activity are desirable. These systems have been appointed as "guided molecular missiles" by Ojima (1), meaning that they should be non-immunogenic, stable in blood circulation, selective and strategically efficient at the site of action.

Even though they are currently still far from being ideal molecular missiles, CNTs have shown interesting properties for cancer nanotechnology (166), especially as a template for multiple functionalizations. Nanotubes are interesting alternatives not only because they have a high mechanical stability and nanometric dimensions, but also because depending on how they are rolled up, they share electronic properties of both metals and semiconductors (167,168). In addition, differently from spherical nanoparticles, they present a large inner volume that could be filled with several biomolecules ranging from small derivatives to proteins (169,170). This offers the advantage to load the inside of CNTs with a drug, while imparting chemical properties through the functionalization of the external walls and thus rendering these tubes water soluble and biocompatible. As reported in Table III, there are 4 main aspects that render this nanomaterial a novel opportunity in cancer therapy, the first of which is represented by the thermal effect: biological systems are known to be highly transparent to 700–1,100 nm NIR light, but SWCNTs show the intrinsic property of displaying strong optical absorbance in this spectral window, and this could be used to trigger endosomal rupture by NIR laser pulses. It was demonstrated *in vitro* that continuous NIR radiation caused cell death because of excessive local heating of carbon nanotubes, while functionalization of CNTs with a folate moiety (FA) guaranteed selectivity towards cancer cells. In fact tumor cells generally present an increased number of folic acid (FA) receptors. Therefore they tend to internalize these functionalized CNTs-FA with a much higher extent, while preventing receptor-free normal cells from destruction (123). Hence, taking into account the transporting capabilities of CNTs, combined with suitable functionalization chemistry and their intrinsic optical properties, we could envisage a new class of novel nanomaterials for cancer therapy with optimized DDS. The limitation of this procedure is that the NIR light is able to penetrate only a few centimeters underneath the surface; therefore, radiofrequency waves were applied since they can intercalate deeply in the body with minimal damage for the surrounding tissues (78). Direct injection of SWCNTs coated with a polyphenylene ethynylene polymer into the liver tumor of rabbits selectively eliminated cancerous cells, thus rendering this technology very promising.

The second methodology is still based on non-covalent functionalization, but it does not make any use of additional radiation. In other words, CNTs are used to form stable complexes for example with small interference RNA (siRNA), which shows a lot of potential in cancer treatment due to its ability to inhibit the gene expression in correspondence of telomerase reverse transcriptase (TERT) (171). Another interesting example is the use of the CNTs-molecule conjugate in the form of pro-drugs for the delivery of the anticancer molecule cisplatin (172): SWCNTs coated with lipid-PEG-platinum(IV), after internalization into the cells, underwent a reduction to platinum(II), thus reconstituting the drug's original activity and avoiding its early inactivation.

In general, non-covalent functionalization seems to create more concerns about potential hazardous effect than covalent binding, since it is less accurate and more difficult to control. It has been already mentioned that the covalent conjugation of

Table III. Applications of CNTs in Nanomedicine ("Nanooncology")

Strategy	Nano-material	Functionalization	Therapeutic effect	Cell line	Reference
Thermal treatment	pSWCNTs	Folic acid + NIR irradiation (700–1,100 nm) on the basis of an intrinsic properties of CNTs	Selective destruction of tumoral cells by excessive local heating	HeLa adherent cell line	(123)
		Water or 1-pyrenebutanoyl succinimide + anti-IGF1R mouse monoclonal and anti-HER2 antibodies + NIR irradiation	Selective destruction of cancer cells	Breast cancer cells	(174,175)
		Polyphenylene ethynylene polymer adsorbed onto CNTs + Radiofrequency	Destruction of malignant tumoral cells by RF-induced thermoablation; cancer cells more sensitive to heat induced damage than normal cells.	HepG2, Hep3B hepatocellular and Panc-1 pancreatic adenocarcinoma cells	(78)
Chemical non-covalent functionalization	pSWCNTs, SWCNTs-ox	1-pyrenebutanoic acid, succinimidyl ester onto CNTs + Streptavidin	–	–	(82)
	pMWCNTs	Amphiphilic (PEG)-based Copolymer + DOX	–	–	(120)
	pMWCNTs	Co-polymer Pluronic F127 + DOX	DOX–MWCNTs complex exhibits enhanced cytotoxic activity compared to both free drug and DOX–pluronic complexes	MCF7 human breast cancer	(121)
	SWCNTs-ox	SWCNTs-CONH-$C_6H_{12}NH_3^+$ and siRNA	Suppression of tumour growth	Cells HeLa cells and LLC, TC-1, and 1H8 tumor cells.	(171)
	SWCNTs	Pro-drug Platinum(IV) via PEG + phospholipid	Conversion to Pt(II) and cell destruction due to improved (6 times) internalization	Testicular carcinoma cell line NTera-2	(172)
Chemical covalent functionalization	MWCNTs	1,3-dipolar cycloaddition + Methotrexate (MTX)	Fast cellular uptake and slightly less toxicity than free drug	Human Jurkat T lymphocytes	(135)
	MWCNTs-ox	Gonadotropin releasing hormone	Death rate of the DU 145 cells was about 82%	DU 145 cells, Hela cells, and L292 cells	(173)
	SWCNTs	Nitrene cycloaddition_C_2B_9 carborane unit	Higher boron concentration in tumor cells	BALB/c mice	(92)
Encapsulation	SWCNTs + DWCNTs	Annealing at 550°C + nano-extraction of Altretamine (HMM)	–	–	(160)
	MWCNTs	Thermal treatment + oxidation + carboplatin	Inhibition cancer cell viability	Human bladder cancer EJ28 cells	(164)

methotrexate with MWCNTs (135) allowed keeping its activity during the whole duration of the experiment (72 h). In addition, Hosmane's group studied how C_2B_{10} carborane cages, incorporated onto CNTs, could be useful in boron capture neutron therapy (BNCT), on the basis of their ability to concentrate especially in tumoral tissues (92). Penetration into malignant cells and their selective destruction was also the interesting result obtained from a hybrid conjugate between CNTs and gonadotrophin releasing hormone (173); it is worth to mention that neither CNTs alone nor this hormone (usually overexpressed at the membrane of several cancer cells) were able to display comparable activity.

Interestingly, the study of CNTs as drug delivery systems has also provided unexpected, incredible insights from the scientists of the University of Delaware (174), who discovered the possibility to induce microscopic explosions of nanotubes in a wide variety of conditions. In this way, they developed a unique "nanobomb" that could literally blow up tumors, as soon as the water inside SWCNTs sheets or on top of the cells evaporates and creates a pressure, which induces CNTs' explosion. Similar to cluster bombs, these tubes started exploding one after another under laser light exposition, carrying great promise as a therapeutic agent able to kill cancer cells on the basis of a selective, localized, and minimally invasive procedure. In addition, once the "nanobombs" burst out, they destroy cancer cells and macrophages can efficiently remove the cell debris and the exploded nanotubes along with it.

Moreover, the latest updates regarding the use of CNTs in "nano-oncology" have suggested a promising role of SWCNTs for detecting cancer-specific proteins; this is feasible due to their dimensions inferior than a single-strain DNA and their arrangement on one layer of carbon atoms. Protein-binding events occurring on the surface of these nanoatomic tubes produce a measurable change in the mechanical and electrical properties, which can be accurately measured; in a similar approach, it has been shown that it is possible to detect the surge in electrical current in nanotubes coated with monoclonal antibodies (MAbs) (175), when cancer cells bind to the Abs. These Abs are specific for insulin-like growth factor-1 receptor (IGF-1R), which is commonly found at high levels on cancer cells. Subsequently it was also measured the change in electrical current through the Ab–nanotube combinations when two different types of breast cancer cells were applied to the devices, demonstrating a direct correlation of the signal with the number of receptors on the surface of tumor cells and the possibility to discriminate among different cell subtypes. These experiments suggest how this method could be used for detection of recurring circulating cancer cells or micrometastases remaining from the originally treated tumor. The advantage would be a cost-effective technique that could diagnose whether cells are cancerous or not in seconds *versus* hours or days required for conventional histology examination. At the moment, the main limitation of the technique is that it may not detect more than one antigen at a time on a single cell, but it paves the way for further developments in the nanomedicine field.

Despite the previously mentioned thriving cases, the extensive use of carbon-based devices suffers from many concerns regarding their safety, their quality and their impact on the environment. Until a few years ago, the development of nanotechnology was mainly restricted to electronics and engineering instruments, representing a rather harmless phenomenon, but recently it has envisaged the possibility of being applied into medicine and biology as well. The first generation of nanomedicines (liposomal preparations) were approved much before a real awareness existed about safety of nanomaterials and their secure use in cancer. Nevertheless, it is important to specify that nanodevices, such as biodegradable polymers or phospholipids, are of a completely different nature from other materials (such as inorganic nanoparticles or carbon nanotubes) that are expected to have deep impact in the next future.

Even though it is too early to say whether the "nanostructures" will wean the world from current limitations presented by DDS, or if they will definitely backfire, there is the general consciousness that the laws of physics and chemistry are pretty different when particles get down to the nanoscale. As a consequence, even substances that are normally innocuous can trigger intense chemical reactions and biological anomalies as nanospecies (176). The potential risk associated with this material is the presence of impurities (e.g. metal catalysts) and the possibility, due to small dimensions of such devices, to escape from the normal phagocytic defenses and deposit into organ and tissues, with unintentional effects on the body. Moreover, the kind of hazards introduced by nanodevices for drug delivery is beyond that posed by conventional chemicals in traditional delivery matrices, and available information concerning the relative environmental and health risks to humans of manufactured nanoparticles or nanomaterials is severely absent and defective. For that reason, there is a deep debate between the desire of introducing nanomaterials in everyday life and the moralistic tendency of blocking the nanotech factory until the risks will be better understood. So far there have been only extreme cases of high optimism and profound skepticism, without a balanced and objective evaluation of the real situation.

CNTs in fact represent an intriguing but ambiguous class of substances, since their shape belongs to both fibres and nanoparticles, and they are often characterized by the presence of metallic components even after their purification. They are classified as "synthetic graphite" by the National Occupational Safety and Health Administration (http://www.osha.gov/dts/chemicalsampling/data/CH_244000.html) on the basis of the same hexagonal/honeycomb pattern. However, such extrapolation might not be protective for the exposure to CNTs, because they show physicochemical properties, which are dependent on size, chemical composition, surface structure, solubility, shape, and aggregation (177). These parameters can modify cellular uptake, protein binding, translocation from portal of entry to the target site, and the possibility of causing tissue injury. At a more general level, one severe drawback is represented by the inability to fabricate structurally and chemically controlled CNTs with identical characteristics in terms of properties and impurities content, and this has also limited their clinical and pharmacological applications. CNTs commonly show different levels of purity, which are strictly dependent on the methods employed for their production. The impurities are essentially made up of residual catalysts and amorphous carbon. If present in high amount, they might enhance the toxicity (178)

and determine unwanted effects. Regarding their dimensions, until now the size cut-off below which nanomaterial is considered surely toxic has not been determined. However, there are at least two main factors that render CNTs potentially unsafe: (i) their large surface area, and (ii) the reactivity of the surface (179,180). It has been observed that the smaller the particles, the more toxic they become, since there is more surface area per mass unit. As a result, any intrinsic toxicity of the surface will deeply affect the toxicological profile of the whole samples. A confirmation of that derived from the study by Sayes *et al.*, who investigated the effect on human fibroblasts (HDF) of some water-dispersible single-walled carbon nanotubes (181): in their experiments they showed that cytotoxicity of compounds decreased significantly with the increased degree of functionalization on the surface but it was also partially attributable to the release, during the biological tests, of the surfactant non-covalently coated on CNTs' surface. This result confirmed that chemical functionalization of CNTs is useful not only to incorporate several moieties or increase dispersibility of the samples, but also to improve the toxicological aspect normally related to this material.

To sum up, there seems to exist a common agreement regarding a concentration-dependent degree (Table IV summarizes the most recent *in vitro* studies and the concentrations used) of toxicity for all types of nanomaterials and an inverse correlation between toxicity and extent of CNTs' functionalization; this last outcome partially justifies the remarkable toxicity reported in many works performed only on pristine, nonfunctionalized CNTs. In particular, carbon nanotubes' needle-like fibre shape has been recently compared to asbestos (182): previous studies in populations exposed to this material showed that the main body of the lung was a target for asbestos fibres, resulting in both lung cancer and scarring of the lungs (asbestosis). Therefore, the analogy between CNTs and asbestos has resulted in huge concerns since CNTs' widespread use may lead to analogue inflammation and formation of lesions known as granulomas. However, these observations do not correspond to samples chemically modified with different chains and molecules; as a confirmation of this, Kostarelos and collaborators (183) have recently shown that intravenously administrated pristine MWCNTs accumulated mainly in lung, liver and spleen, while the functionalized tubes tended to persist much less in tissues and organs, with an accumulation proportional to the degree of functionalization but independent from the characteristic of the attached groups. The evidence could be explained by the fact that pCNTs are extremely hydrophobic and difficult to disperse in aqueous milieu owing to the van der Waals forces leading to aggregation in bundles, while functionalization allows to obtain more individual tubes. Another parameter that was found to be involved in the toxicity profile of CNTs is their length. Sato and coworkers separated MWNT of 220 and 825 nm, using controlled strong acid conditions (184). During a short incubation time, clusters of both samples resulted to be surrounded by macrophages as a consequence of the activation of innate immunity. The shorter tubes displayed a lower inflammatory response, even though in both cases, no severe effects, such as necrosis or degeneration, were observed around CNTs throughout the experimental period of 4 weeks.

With regard to biodistribution, so far there are only a few studies on *f*-CNTs (72,185,186). For the purpose, water-soluble, SWCNTs were functionalized with the chelating agent diethylenetriaminepentaacetic acid (DTPA) and labeled with indium (^{111}In) for imaging purposes (72). Subsequently, intravenous administration of these *f*-SWCNTs followed by radioactivity tracing indicated that the tubes were not retained in any of the reticulo-endothelial system organs (liver or spleen). Since only water-soluble tubes were employed, neither toxic side effects nor mortality were reported; in the most of the cases (72,186), nearly all the tubes were excreted through the renal pathway in the form of intact tubes in the urine, without any remarkable tissue damage even at high concentrations of tubes. The next steps for this study is to prolong the blood circulation of CNTs, since a rapid blood clearance and half-life (3 h) of *f*-SWCNTs has been observed; this will give them enough time to get to a target tissue, thus enhancing the pharmaceutical development of functionalized CNTs for drug delivery.

CONCLUSIONS

Considering the different DDS that are currently employed at the nanoscale level, together with the intrinsic and unique properties of carbon nanotubes and the successful examples of their application in diversified contexts, we could conclude that CNTs are promising materials especially for potential multimodality cancer therapy and imaging. While pristine nanotubes are completely insoluble and present several impurities that limit their biomedical applications, many strategies have been successfully adopted to overcome these problems and to offer better pharmacological profiles. Functionalized CNTs permit to incorporate simultaneously several drugs, targeting agents and even metals (e.g. iron) able to induce hyperthermia and thus improve the therapeutic activities. Preliminary studies have shown that the selective chemical ligation between CNTs and a drug candidate is mainly based on covalent bonds, therefore the delivery relies on the cellular uptake of the entire CNT-drug conjugates. On the other side, the physical adsorption of bioactive agents on the surface of the tubes has demonstrated an extensive but less accurate drug delivery. In both cases, though varying accordingly to the extent of their functionalization, CNTs have demonstrated to improve the effects and reduce toxicity of several drugs, thus strengthening the idea that a chemical modification at their external or internal surfaces could achieve intriguing results and advance their role as DDS.

In addition, their natural huge aspect-ratio allows them to behave like nanoneedles that do not disrupt the integrity of external membranes during their cellular uptake. At the same time, their extraordinary strength has shown to preserve their structure, as demonstrated by their excretion as intact tubes after intravenous administration in mice.

However, an important aspect to consider is that many pharmaceutical scientists are using nanomatrices to reduce toxicity and undesired effects of drugs but up to recently they have not realized that carrier systems themselves may impose relevant risks. The toxicology profile of nanoparticulate matter differs from that of classical substances as the contact with these materials may vary from a rather high local

Table IV. Effect of CNTs at Different Concentrations in Several Cell Lines

No.	Article title	Journal/Publish date of article
1	*In vitro* toxicity of single walled carbon nanotubes on human A549 lung cells.	Toxicology *In Vitro* Volume 21 Issue 23 April 2007
2	Multi-walled carbon nanotubes induce T lymphocyte apoptosis	Toxicology Letters 160 2006 (121–126)
3	Cellular toxicity of carbon based nanomaterials	Nano Letters 2006 Volume 6 No. 6 1121–1125
4	Functionalised Carbon Nanotubes are Non-cytotoxic and preserve the functionality of Primary Immune Cells	Nano Letters 2006 Volume 6, No. 7 1522–1528
5	Biological interactions of functionalised single-wall carbon nanotubes in human epidermal keratinocytes	
6	Chemical modification of SWCNT alters *in vitro* cell-SWCNT interactions	Journal of Biomedical Research Part A Volume 76, Issue 3, Pg 614–625
7	Cytotoxicity of single-wall carbon nanotubes on human fibroblasts	Toxicology *in vitro* 20 (2006) 1202–1212
8	Impact of carbon nanotube exposure, dosage and aggregation on smooth muscle cells	Toxicology Letters Volume 169, Issue 1, 28 February 2007, Pg 51–63
9	Effect of single wall carbon nanotubes on human HEK293 cells	Toxicology Letters Volume 155, Issue 1, 15 January 2005, Pages 73–85
10	The degree and kind of agglomeration affect carbon nanotube cytotoxicity	Toxicology Letters 168 (2007) 121–131
11	Investigation of the cytotoxicity of CCVD (catalytic chemical vapour deposition) carbon nanotubes towards human umbilical vein endothelial cells	Carbon 44 (2006) 1093–1099
12	*In-vitro* studies of carbon nanotubes biocompatibility	Carbon 44 (2006) 1106–1111
13	Spectroscopic analysis confirms the interaction between single walled carbon nanotubes and various dyes commonly used to assess cytotoxicity	Carbon 45 (2007) 1425–1432
14	Effects of fullerenes and single-wall carbon nanotubes on murine and human macrophages	Carbon 44 (2006) 1100–1105
15	Functionalization density dependence of single-walled carbon nanotubes cytotoxicity *in vitro*	Toxicology Letters Volume 161, Issue 2, 20 February 2006, Pages 135–142
16	Cytotoxicity assessment of some carbon nanotubes and related carbon nanoparticle aggregates and the implications for anthropogenic carbon nanotube aggregates in the environment	International Journal of Environment Research and Public Health ISSN 1660–4601 2005
17	Influence of length on cytotoxicity of multi-walled carbon nanotubes against human acute monocytic leukaemia cell line THP-1 *in vitro* and subcutaneous tissue of rats *in vivo*	Molecular Biosystems 2005, 1, 176–182
18	Multi-walled carbon nanotube interactions with human epidermal keratinocytes	Toxicology Letters Volume 155, Issue 3, 15 March 2005, Pages 377–384
19	Carbon nanotube biocompatibility with cardiac muscle cells	Institute of Physics Publishing Nanotechnology 17 (2006) 391–397
20	*In-vitro* toxicity assessment of single and multi-walled carbon nanotubes in human astrocytoma and lung carcinoma cells	Toxicology Letters 172S (2007) S1-S240 (Full text not available)

Type of carbon nanotubes		
Single walled (SWCNTs)	Multi walled (MWCNTs)	Cell line used
Non-functionalized		Human A549 lung cells
	1. Oxidised 2. Pristine	Jurkat T-leukemia cells
	1. Pristine 2. Oxidised	Initial: three different human lung-tumour cell lines, H596, H446 and Calu-1. Principal experiment: H596
1. 1,3-dipolar cycloaddition reaction 2. Oxidationamidation methodology		1. Purified B 2. T lymphocytes 3. Macrophages (obtained from the spleen, lymph nodes, and peritoneal cavity of BALB/c mice)
Purified CNTs + AHA (6-aminohexanoic acid) in DMF. (covalent bond formation)		Neonatal human epidermal keratinocytes
1. Pristine 2. Purified 3. Functionalised with glucosamine. (amide bond formation between glucosamine and SWCNTs)		3T3 mouse fibroblasts
Refined SWCNTs	Refined MWCNTs	Human dermis fibroblasts
Purified and acid treated		Rat aortic smooth muscle cells
Not stated whether it is pristine or purified		Human embryo kidney cells (HEK293)
Four different fractions: CNT-raw material CNT-agglomerates CNT-bundles CNT-pellet		Mesothelioma cell line (MSTO-211H)
Three different samples, each synthesised by different catalytic chemical vapour deposition		Human umbilical vein endothelial cells (HUVEC)
	High purity MWNTs coated on polysulfone films	1. Human osteoblastic line hFOB 1.19 ATCC CRL-11372 2. Human fibroblastic line HS-5 ATCC CRL-11882
Pristine SWCNTs		Human alveolar carcinoma epithelial cell line (A549)
Purified SWCNTs		1. Murine macrophage cells (J 774 cell line) 2. Human monocytes derived macrophages
Four water-dispersible SWNT samples: 1. SWCNT-phenyl-SO3H 2. SWCNT-phenyl-(COOH)2 3. SWCNT in 1% Pluronic F108 4. SWCNT-phenyl-SO3Na		Human Dermal Fibroblasts (HDF)
Pristine SWNT sample	Two different MWCNT samples from two companies.	Murine alveolar macrophages (RAW267.9 cells)
	Purified, acid-treated MWCNTs of two different lengths: 1. 220 nm 2. 825 nm	Human acute monocytic leukaemia cell line (THP-1)
	Self-prepared MWCNTs	Human Epidermal Keratinocytes (HEK)
Highly purified SWCNTs		Cardiac muscle cell lines (rat cell line H9c2)
SWCNTs	1. MWCNTs 2. MWCNT-COOH 3. MWCNT-NH_2	Human astrocytoma D384-cells Lung carcinoma A549-cells

Table IV. (Continued)

No.	Viability	Concentration (if applicable)
1	Low acute toxicity	Concentration range used: 1.56–800 μg/ml
2	Oxidised: loss of >80% Pristine: loss of less than 50%	400 μg/ml 400 μg/ml
3	Pristine: Cell viability decreased but less pronounced than that of the other CBN materials used in experiment Acid treated: significant increase in toxicity compared to the pristine MWCNTs.	0.02 μg/ml
4	No significant toxicity for all three cell lines	10 μg/ml
5	Significant decrease in viability from 0.00005 to 0.05 mg/ml	Concentration range used: 0.00000005 to 0.05 mg/ml
6	1. AP NT: At the lowest tested concentration, 55% cell viability 2. Purified and glucosamine functionalised: dose-dependent decrease in viability but less toxic than AP-NTs	Concentration range used: 0.001–1.0% (wt/vol)
7	Refined SWCNTs exhibited the most toxic effect (at 25 μg/ml)	Concentration range used: 0.8–100 μg/ml
8	Significant effect on cell viability	Concentration range used: 0.18 and 0.22 mg/ml
9	SWCNTs inhibit the proliferation of HEK293 cells.	Concentration range: 0.78125 μg/ml to 200 μg/m1
10	All CNTs fractions were able to significantly decrease cell activity and proliferation in a dose-dependent way	Concentration range used: 7.5–30 g/m1
11	Non-toxic	Concentration range: 100% to 1% (volume by volume) Volume used: 100 μl
12	Good biocompatibility	Not applicable
13	Toxicity was observed	Concentration range used: 0.00156–0.8 mg/ml
14	Low cytotoxicity	Concentration range: 30–60 μg/ml
15	Covalent functionalisation reduced HDF cytotoxic response overall, limited impact on cell viability	Concentration range: 3 μg/mL–30 mg/mL
16	Significant cytotoxic effect. Induction of cellular death at a threshold of 2.5 μg/mL	Concentration range used: 10 μg/mL with 11 doubling concentrations subsequently
17	Cytotoxic effects was based on the production of TNF-α Both 220-CNTs and 825-CNTs possess induction activity towards macrophages	Concentration range used: 5–50 ng/ml
18	HEK viability assessed by the NR assay slightly decreased in a dose-dependent manner at 24 and 48 h (data not shown) after exposure to the nanotubes.	Concentration range used: 0.1, 0.2, and 0.4 mg/ml
19	SWCNTs can be seeded with cardiomyocytes without affecting cell viability	Concentration range used: 0.2 mg/ml
20	MTT results revealed a strong cytotoxicity. Calcein/PI staining did not confirm MTT cytotoxicity in both cell lines	Concentration range: 0.1–100 μg/mL

exposure in the lungs to a low or negligible contact with other organ systems after inhalation. Taking into account these observations, it should be stressed that more studies are indispensable for demonstrating the real properties of CNTs as potential DDS, in particular regarding their toxicity and most importantly about the delivery of drugs covalently or non covalently linked to the tubes. Therefore, at this stage no categorical statement can be made about the effectiveness of nanotubes; differences in the starting materials, in the chemical procedures, in the incorporated molecules, in the purity of the samples and in the doses used during the experiments do not allow any exhaustive conclusion about

their mechanism of cellular uptake and their toxicological profile. The most of the data reported in this manuscript are at their infancy, in form of proof-of-concept studies, therefore they do not present systematic, preclinical therapeutic results. As a consequence, we are not yet in the situation to decide whether carbon nanotubes are entirely safe molecular missiles or surely dangerous asbestos' analogues until further investigations will provide fundamental insights."

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