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Review

Multifunctionalized carbon nanotubes as advanced multimodal nanomaterials for biomedical applications

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Abstract

The increasing importance of nanotechnology in the field of biomedical applications has encouraged the development of new nanomaterials endowed with multiple functions. Novel nanoscale drug delivery systems with diagnostic, imaging and therapeutic properties hold many promises for the treatment of different types of diseases, including cancer, infection and neurodegenerative syndromes. Functionalized carbon nanotubes (CNTs) are one of the most recent type of nanomaterial developed in biomedicine as they can be designed and imparted with multimodal capabilities. Indeed, the possibility of inserting different functionalities on CNTs is opening the possibility to exploit them on new strategies that combine diagnosis with improved therapeutic efficacies. In this review, we describe the different approaches that have been recently developed to generate multifunctionalized CNTs for biomedical applications. In particular, covalent and non-covalent double and triple functionalization methods are discussed, putting in evidence their use *in vitro* and *in vivo* and highlighting the advantages and the drawbacks of these new systems. Preclinical studies have demonstrated that multifunctional CNTs are highly promising when combining diagnostic, imaging and therapeutic modalities.

Keywords: biomedicine; carbon nanotubes; nanomaterials.

1. Introduction

Carbon nanotubes (CNTs) [1] are hollow cylindrical objects with unique structural, mechanical [2] and electronic [3] properties. They can be classified into two types: single-walled carbon nanotubes (SWCNTs), which consist of a single layer of graphene sheet rolled up into a tubular form, and multi-walled carbon nanotubes (MWCNTs), which are comprised of multiple layers of concentric cylinders with a diameter of up to 100 nm and a spacing of approximately 0.34 nm between the adjacent layers (Figure 1). The walls of CNTs are made up of a hexagonal lattice of sp^2-hybridized carbon atoms analogous to the atomic planes of graphite.

Because of the exceptional combination of their mechanical, electronic and thermal properties, CNTs are considered as unique materials, with very promising future applications, especially in the fields of nanoelectronics [4], composite materials [5] and nanomedicine [6]. However, there is still a long way to translate the promising properties of CNTs into real applications. This is mainly due to the difficulty in manipulation because of the poor solubility in all solvents and the high tendency to aggregate. To overcome this obstacle and to fully exploit the CNT characteristics, chemical functionalization is extremely important as it increases their processability and allows combining their remarkable properties with those of other classes of materials. The development of reliable derivatization methods has provided an additional impetus to extend the scope of their applications. Current approaches include defect and covalent sidewall functionalization, as well as non-covalent exo- and endohedral functionalization [7]. Covalent grafting of molecules to CNTs is possible via oxidation with concentrated acids [8], arylation [9], cycloaddition [10] and other reactions requiring highly reactive species. The covalent attachment of functional groups results in a change of carbon hybridization from sp^2 to sp^3, leading to a possible partial loss of conjugation and the introduction of defects in the nanotube structure with consequences for electron-acceptor and/or electron transport properties [11]. Non-covalent chemistry has the advantage to preserve the electronic properties of CNTs. The nanotube surface can be modified by adsorption or wrapping of polynuclear aromatic compounds [12], surfactants [13], polymers [14] or biomolecules [15] via van der Waals forces or π-π interactions. However, non-covalent bonds are susceptible to environmental factors, such as pH and salt concentration, and are less stable in general than covalent bonds, leading to a probable premature release in the case of uses as support for therapeutic agent delivery. Multifunctionalization of CNTs can be necessary to impart multimodalities and extend the scope of potential applications of CNTs. In particular, for the development of future multipotent therapeutic constructs, it is of interest to use CNTs as multimodal drug delivery. In the biomedical domain and in nanomedicine [16], CNTs have become the focus of much attention. The immobilization of different types of bioactive molecules including peptides [17], proteins [18], nucleic acids [19] and small drugs [20], by covalent or non-covalent bonds, might find interesting applications for the delivery of

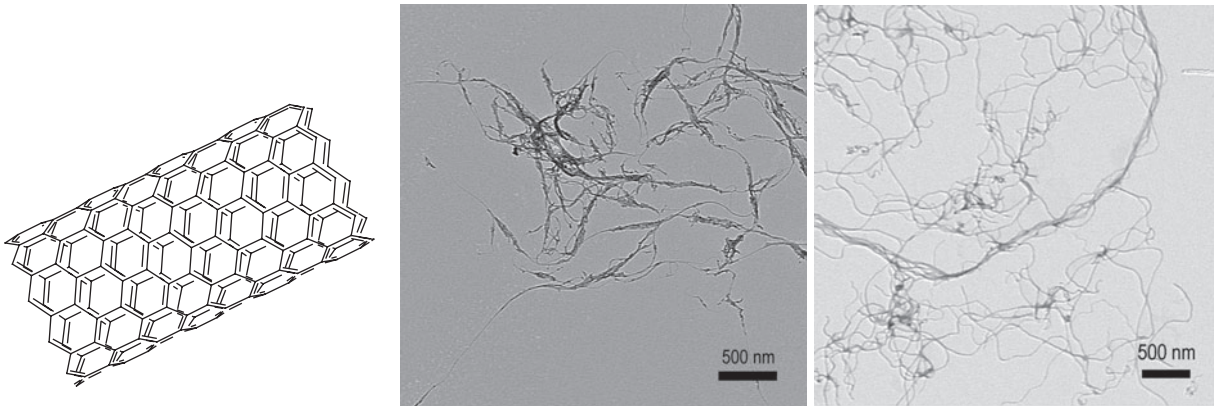

Figure 1 Schematic representation of an opened section of a single-walled carbon nanotube (left); transmission electron microscopy images of SWCNTs (middle) and MWCNTs (right).

therapeutic molecules [21], for the modulation of molecular and cellular interactions [22], for DNA transfer [23] and RNA silencing [24], and in diagnostics [25]. The development of nanocarriers with targeting and imaging capability is essential in the treatment of many diseases. Targeting of therapeutic agents to the desired site of action by conjugation to antibodies or ligands of receptors overexpressed at the surface of pathogenic cells is of high importance to increase the therapeutic efficiency and reduce side effects. In addition, the attachment of a tracking probe allows imaging and localizing the CNT conjugates [26]. In this review, we will focus on the strategies developed for the double and triple functionalization of CNTs using covalent and/or non-covalent approaches. The advantages brought by such highly advanced CNT conjugates will be also highlighted, in particular for biomedical applications.

2. Covalent double functionalization of carbon nanotubes

In the past decade different covalent approaches for CNT functionalization have been exploited to develop carbon nanotubes as a new drug delivery system. The strong covalent bonding of drugs onto CNTs is fundamental to avoid their release and metabolization before reaching the pharmacological target. In this context, the double covalent approach permits the exploitation of CNTs as biological vectors for different tasks at the same time, by combining an imaging probe or a targeting molecule with a therapeutic agent. In this way, it is possible to study both *in vitro* and *in vivo* the localization and the drug release properties of these nanoconjugates or to specifically carry low-selective pharmacophores into the desired tissue. A further elaboration of these systems by the introduction of cleavable linkers between the drug and the CNTs will allow the release of the drug by specific enzymes only when the target tissue is reached. At present, the different functionalization techniques exploited for the preparation of covalently double functionalized CNTs can be classified into five general approaches described below.

2.1. Photoactivation approach

The first example of covalent double functionalization of CNTs has been reported in 2005 by Dai and coworkers [27]. The authors showed a simple and effective method to produce asymmetrically tip-functionalized MWCNTs, based on the grafting of different chemical reagents at the two ends of a prealigned CNT film. As first step of this procedure, the two edges of a vertically aligned CNT film were sequentially floated on two different photoreactive solutions containing 3 -azido-3 -deoxythymidine (AZT) [28] and perfluorooctyl iodide (F) [29], respectively. Only one side of the nanotube film was in contact with the photoreactive solution and exposed to UV light each time, which induced the selective link of the two different organic moieties at the opposite tips of the CNTs, producing in this way a new type of hydrophilic/hydrophobic MWCNTs (AZT-CNT-F). The authors showed that, after destroying the CNT alignment by sonication, the AZT-CNT-F nanotubes were able to self-assemble again at the interface of a polar/apolar solvent system.

2.2. 1,3-Dipolar cycloaddition/amidation approach

In 2005, using a different approach, two different and orthogonal functionalizations have been applied by our group to prepare doubly functionalized MWCNTs [30]. This method allowed to link a fluorescent probe such as fluorescein and the antifungal drug amphotericin B (Figure 2, Approach A). The first synthetic step consisting of the oxidation of CNTs was necessary to decrease the length of the pristine material and to increase the number of defects on CNTs. The carboxylic groups obtained by the oxidation process were subsequently modified by amidation, whereas on the sidewall of oxidized CNTs 1,3-dipolar cycloaddition of azomethine ylides was performed. Triethylene glycol (TEG) amine moieties with different protecting groups [*t*-butyloxycarbonyl (Boc) and phthalimide] were introduced on the CNTs, allowing the orthogonal deprotection needed for further heterofunctionalization with the imaging probe and the therapeutic agent. The fluorescent conjugates obtained in this way permitted to study

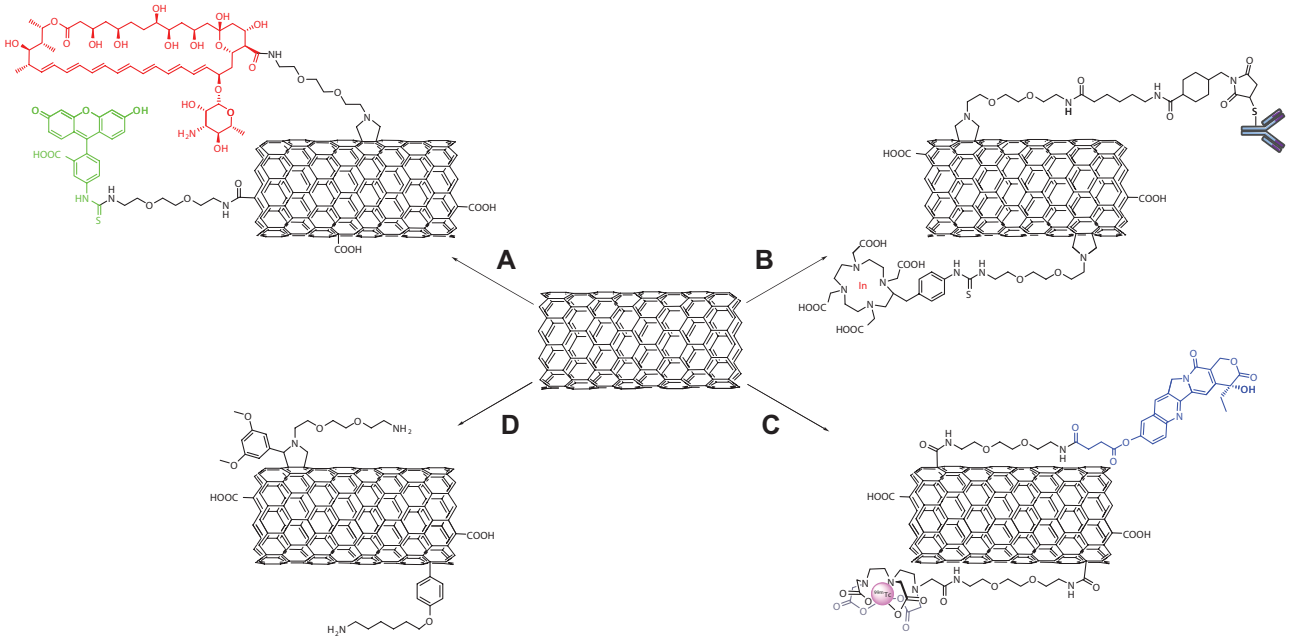

Figure 2 Different covalent approaches for the double functionalization of CNTs.

the intracellular trafficking of CNTs by fluorescence and confocal microscopy, highlighting the localization of functionalized CNTs in the perinuclear region. The potent antifungal activity of amphotericin B covalently bound to CNTs was preserved or even increased for certain strains [31].

A similar approach was exploited by Chen et al. [32] and by Villa et al. [33] in 2008. In the first study, SWCNTs were double functionalized with both a tumor-targeting and a prodrug module to obtain a drug delivery system endowed with high selectivity for tumor cells [32]. Biotin was chosen as the tumor-targeting module, due to overexpression of its receptor on the surface of certain types of cancer cells. A taxoid compound was used as the prodrug, inserted via a linker designed to decrease the undesired toxicity of the drug and to be efficiently cleaved once in the cancer cells by an endogenous reductant system such as glutathione. After performing 1,3-dipolar cycloaddition and amidation reactions on CNTs, biotin was linked to the CNTs followed by the taxoid, modified with fluorescein to track the cellular uptake, and conjugated to the nanotubes via a cleavable linker. Cellular studies conducted on different cell lines showed a higher CNT internalization in cells overexpressing biotin receptor, following an endocytosis mediated mechanism of penetration. A perturbation in the microtubule organization was observed, which is indicative of an intracellular release of the drug because of the sensitive linker.

Villa et al. [33] explored the synthesis of double functionalized CNTs bearing an oligonucleotide (ODN) sequence and a radiolabeling compound or a targeting peptide. In this study, an ODN able to specifically hybridize a complementary ODN was covalently bound to oxidized CNT tips. After performing 1,3-dipolar cycloaddition, the terminal amino groups were either functionalized with DOTA (1,4,7,10-tetraazacyclododecane-1,4,7,10-tetracetic acid) and complexed to indium to follow the organ biodistribution of the conjugate, or with the arginine-glycine-aspartic acid (RGD) peptide, which selectively targets tumor cells. Cellular studies confirmed the specific targeting and binding to tumor cells.

Our group [34] devised a new synthetic strategy to enhance the anticancer activity of methotrexate (MTX) by exploiting the ability of CNTs to cross the cellular membrane and deliver the anticancer drug by selective enzymatic cleavage. Two cleavable linkers were used to bind MTX to CNTs: (i) a tetrapeptide, selectively sensitive to proteases overexpressed in cancer cells and (ii) 6-hydroxyhexanoic ester, a spacer widely used in prodrug for its esterase sensibility. Oxidized CNTs subsequently modified by the 1,3-dipolar cycloaddition reaction were functionalized with sulforhodamine at the carboxylic groups mainly localized at the tips and with a MTX-linker moiety on the sidewall. The conjugates were efficiently internalized in human breast carcinoma (MCF-7) as assessed by confocal microscopy. An increased cytotoxicity was observed for the CNT-MTX conjugates with the cleavable linkers in comparison to the free drug and the CNT-MTX without the linker. In particular, the CNT hybrids with the tetrapeptide linker resulted more active than the other constructs.

2.3. Double 1,3-dipolar cycloaddition approach

In 2006, our group [35] had already functionalized CNTs with MTX and a fluorescent probe using a different approach. In this case, 1,3-dipolar cycloaddition of azomethine ylides was performed using contemporaneously two TEG modified amino acids orthogonally protected with Boc and phthalimide groups. Firstly, the phthalimide moiety was selectively removed to bind fluorescein isothiocyanate (FITC) and the CNTs were subsequently treated with a HCl solution to

deprotect the second amine blocked by the Boc group, allowing the introduction of MTX. In this way, we showed a novel easy strategy to achieve double functionalized CNTs using 1,3-dipolar cycloaddition.

Exploiting this strategy, McDevitt et al. [36] and Ruggiero et al. [37] functionalized SWCNTs with a chelating agent, DOTA or desferrioxamine B (DFO), and a therapeutic antibody. In the study of McDevitt et al. (Figure 2, Approach B), SWCNTs mono-functionalized with DOTA complexed to the radioactive indium mainly accumulated in the kidney, spleen, liver and bones [36]. However, the conjugation of a therapeutic antibody (Rituximab or Lintuzumab) on the ^{111}In/DOTA-SWCNTs allowed to prove the specific targeting of tumor cells *in vitro* and *in vivo*. Similarly, Ruggiero et al. [37] used two different chelating agents to link the radionuclides ^{225}Ac or ^{89}Zr to CNTs. Both radionuclides can be used for *in vivo* imaging of cancer cells as ^{225}Ac possesses radiotherapeutic properties, whereas ^{89}Zr can be used as a positron emission tomography (PET) agent. 1,3-Dipolar cycloaddition using a TEG amino acid was performed on SWCNTs and the free amino groups introduced on the nanotube surface were partially functionalized with reactive hydrazinopyridine (HNH) moieties and partially with DOTA or DFO to complex ^{225}Ac or ^{89}Zr, respectively. Subsequently, a targeting antibody (E4G10) able to specifically bind angiogenic endothelial cells expressed in tumors was linked to the nanotube sidewall on the HNH moieties. The *in vivo* studies showed the efficacy of the ^{225}Ac-CNT conjugates in decreasing the tumor volume after irradiation of the vessels, whereas PET images proved the rapid and specific accumulation of ^{89}Zr-CNT into the tumor.

2.4. Double amidation approach

An alternative procedure to doubly functionalize CNTs relies on a double amidation approach using oxidized CNTs. The oxidation process is generally performed in a mixture of highly oxidizing acids able to shorten the carbon framework of CNTs and introduce carboxylic groups located prevalently on their termini. Subsequently, the acid residues are activated through the conventional synthetic techniques based on the formation of acyl chlorides or by using coupling agents, such as 1-(3-(dimethylamino)propyl)-3-ethylcarbodiimide hydrochloride (EDC) to perform the final coupling with amine derivatives.

Bhirde et al. [38] and Wu et al. [39] have recently reported the possible use of oxidized CNTs as an anticancer drug delivery system after double functionalization via tip amidation with an anticancer agent and a targeting module or an imaging probe. In the study of Bhirde et al. [38], oxidized SWCNTs were first functionalized via amidation with the epidermal growth factor (EGF), whose receptor is overexpressed in most squamous cancer cells. A second molecule, an anticancer drug (cisplatin) or a fluorescent quantum dot (QD) was linked to CNTs via complexation or amidation, respectively. The localization of EGF-QD-CNT conjugates was investigated *in vitro* on HN13 cells overexpressing the EGF receptor (EGFR) and *in vivo* using the HNSCC xenograft model in which HN12 cells overexpressing EGFR were used to induce tumor in mice. The results showed an efficient interaction between EGF and EGFR mediating the internalization of CNTs. In particular, the *in vivo* experiments showed the rapid accumulation of the targeting bioconjugates in the tumor and the rapid clearance of the QD-CNT control (lacking EGF) without any organ accumulation. Further *in vivo* experiments demonstrated the anticancer efficiency of the cisplatin-EGF-CNT conjugates as mice showed considerable regression in tumor growth, whereas the mice administered with the CNT control displayed a clear increase of the tumor volume. From these results, it is evident that EGF-modified SWCNTs can selectively and efficiently target and kill squamous cancer cells.

Wu et al. [39] have developed a drug delivery system consisting of MWCNTs bearing the antitumor agent 10-hydroxycamptothecin (HCPT) and an imaging probe (Figure 2, Approach C). The synthesis of the conjugates began with the amidation of the carboxylated tips of CNTs with a TEG-amino chain, which was further functionalized with HCPT and with FITC or ^{99m}Tc-DTPA (diethylenetriaminepentaacetic acid). An ester linker was introduced between CNTs and HCPT to permit the release of the drug only in the intracellular environment. The *in vitro* release tests confirmed the stability of the HCPT-CNT conjugates at pH 7.4 and 5.0 and showed the release of HCPT in the presence of fetal bovine serum. The FITC-HCPT-CNT conjugates were able to cross the cell membrane of human gastric carcinoma MKN-28 cells and to localize into the cytoplasm. A significant improvement of cytotoxicity was observed for HCPT-CNTs in comparison to HCPT alone. The CNT conjugates bound to ^{99m}Tc-DTPA were injected subcutaneously in hepatic H22 tumor-bearing mice to study the biodistribution at different times by single-photon emission computed tomography (SPECT) and gamma scintillation counting. The results confirmed a rapid distribution of CNTs throughout most tissues including the tumor area. Histological studies showed necrotic zones in tumor slices from mice treated with HPCT-CNTs for 16 days, confirming the efficacy of the drug. The lack of cell mortality in other organs, such as liver or spleen, despite the presence of CNTs, confirmed the low toxicity of CNTs.

2.5. Other double functionalization approaches

Other methods have been developed for the double functionalization of CNTs, including the combination of 1,3-dipolar cycloaddition of azomethine ylides and arylation using diazonium salts. Microwave (MW) heating has been exploited to perform this type of multiple heterofunctionalization (Figure 2, Approach D) [40, 41]. The high number of functionalities introduced by arylation and the possibility to perform the 1,3-dipolar cycloaddition in solvent-free conditions seem to be a highly promising multimodal tool for the generation of new CNT conjugates. MW irradiation generally allows remarkable reaction accelerations and yield improvements. Under MW irradiation, the 1,3-dipolar cyloaddition of azomethine ylides [42] and arylation [43] were subsequently performed using different precursors and reagents. Functionalization of CNTs via the use of diazonium salts was initially developed by the group of Tour [9]. Rubio et al. [41] demonstrated an

improvement of the reaction yield under MW conditions in comparison to the classical heating procedure.

Using the same approach, the possibility to double functionalize carbon nanohorns (CNHs), a new nanostructured form of SWCNTs [44] was reported [45]. The combination of the 1,3-dipolar cycloaddition of azomethine ylides in the absence of solvent and the subsequent addition of diazonium salts in water permitted to obtain highly soluble materials. Moreover, the presence of orthogonally protected groups offers possibilities to multifunctionalize CNHs with different molecules for applications in the fields of clean energy technologies, biology and medicine.

Two novel methods for the preparation of water-soluble SWCNTs were reported by Tour and coworkers [46]. In the first approach, SWCNTs were functionalized with two different diazonium salts in a two-step sequence. As alternative to this strategy based on functionalization by repetitive arylation protocol, a combination of amidation with a polyethylene glycol (PEG)-amine and arylation reactions was developed to double functionalize oxidized SWCNTs.

The possibility to covalently multifunctionalize CNTs with different types of organic moieties displays remarkable biological advantages in comparison to their monofunctionalized homologs, due to the possibility of simultaneously performing different tasks *in vitro* and *in vivo*.

3. Non-covalent double functionalization of carbon nanotubes

Non-covalent double functionalization of carbon nanomaterial has been highly investigated over the past years to generate CNT conjugates bearing targeting or imaging moieties. In this context, physical adsorption of polymers, such as PEG, onto the sidewall of CNTs was widely used to improve the biocompatibility and water dispersibility of CNTs, prerequisites for the formulation of new drug delivery platforms.

Dai and coworkers [47] proposed one of the first examples of non-covalent double functionalization of SWCNTs for biological applications. Indeed, the authors investigated the *in vivo* biodistribution and tumor targeting ability of SWCNTs non-covalently functionalized with phospholipid-polyethyleneglycol (PL-PEG) chains bringing at their termini the chelating agent DOTA for ^{64}Cu radionuclide complexation and the RGD peptide for targeting of integrin $\alpha_v\beta_3$-positive tumors (Figure 3, Approach A) [48]. Nude mice bearing subcutaneous U87MG human glioblastoma tumor model were intravenously injected with ^{64}Cu-labeled SWCNT-PEG. *In vivo* PET and *ex vivo* radioactivity measurements revealed that SWCNTs with a PEG chain of 5400 Da molecular weight exhibited low uptake by the reticuloendothelial system and long blood circulation half-life. *Ex vivo* Raman spectroscopy was used to directly detect the presence of nanotubes in the various mice tissues and to confirm a high tumor uptake.

In 2007, the same group [49] introduced the new concept of "functionalization partitioning" of SWCNTs. This concept consisted of chemically attaching different and multiple species, such as drugs, targeting tags and fluorescent dyes, on the nanotube surface. It was demonstrated by UV-Vis-NIR and fluorescence spectroscopy that water-soluble SWCNT-PL-PEG bind various aromatic molecules, including anticancer drugs [doxorubicin (DOX) and daunorubicin (DAU)] and fluorescent molecules, non-covalently via π-stacking and hydrophobic interactions (Figure 3, Approach B). To target the DOX delivery for selective destruction of cancer cells, the RGD peptide was conjugated to the terminal amine groups of PL-PEG chains on SWCNTs. The obtained data suggest that the nanotube surface area can be exploited for targeted drug and/or dye delivery, with the possibility of their release in tumor acidic environments.

In a subsequent study, Dai and coworkers [50] investigated the *in vivo* pharmacokinetics and biodistribution of the SWCNT-DOX conjugates compared with free DOX, after injection into SCID mice bearing Raji lymphoma xenograft tumors. Measurements of DOX concentration in blood and harvested organs by fluorescence spectroscopy showed prolonged blood circulation and higher tumor uptake, respectively, for the SWCNT-DOX formulation. The *in vivo* therapeutic efficacy of DOX-loaded nanotubes was also investigated and compared to free DOX and DOXIL [51] (a pegylated liposomal DOX formulation). Mice treated with the SWCNT-DOX conjugates showed a greater inhibition of tumor growth than free DOX, but inferior than DOXIL at the equivalent dose. In contrast to the treatment with DOX and DOXIL that resulted in a significant decrease in mouse body weight and an increase in mortality, the SWCNT-DOX formulation did not exhibit toxic effects. Thus, CNTs have great potential as an effective drug delivery system that could improve therapeutic efficacy and reduce systemic toxicity of the free drug.

Based on a similar method, Zhang et al. [52] reported the loading of polysaccharide-modified SWCNTs with DOX for targeted drug delivery with acidic pH-controlled release to cancer cells (Figure 3, Approach C). Both the DOX loading efficiency and release rate were finely tuned by varying the ratio of sodium alginate (ALG) and chitosan (CHI) polysaccharides on the nanotube surface. Moreover, folic acid (FA), a targeting agent for many tumors [53], was additionally attached to the SWCNTs via the CHI chains to selectively deliver DOX to cancer cells. Fluorescence and transmission electron microscopy (TEM) images together with cell viability studies of HeLa cells treated with SWCNT-CHI-FA/ALG-DOX suggested that the nanotubes were internalized by the FA receptor-mediated pathway and accumulated into the lysosomes, where the low pH induces the release of DOX, which migrates into cell nucleus and exerts its cytotoxic effect.

In another demonstration of a bifunctional approach and a non-covalent supramolecular assembly of aromatic molecules on CNTs, our group [54] demonstrated that copolymer-coated MWCNTs were able to form complexes with DOX and enhance its cytotoxic activity. Pristine MWCNTs were dispersed in water using the tri-block copolymer Pluronic F127 and then mixed with DOX at various mass ratios (Figure 3, Approach D). A sharp decrease in the intensity of DOX fluorescence was observed due to the static quenching of DOX absorbance *via*

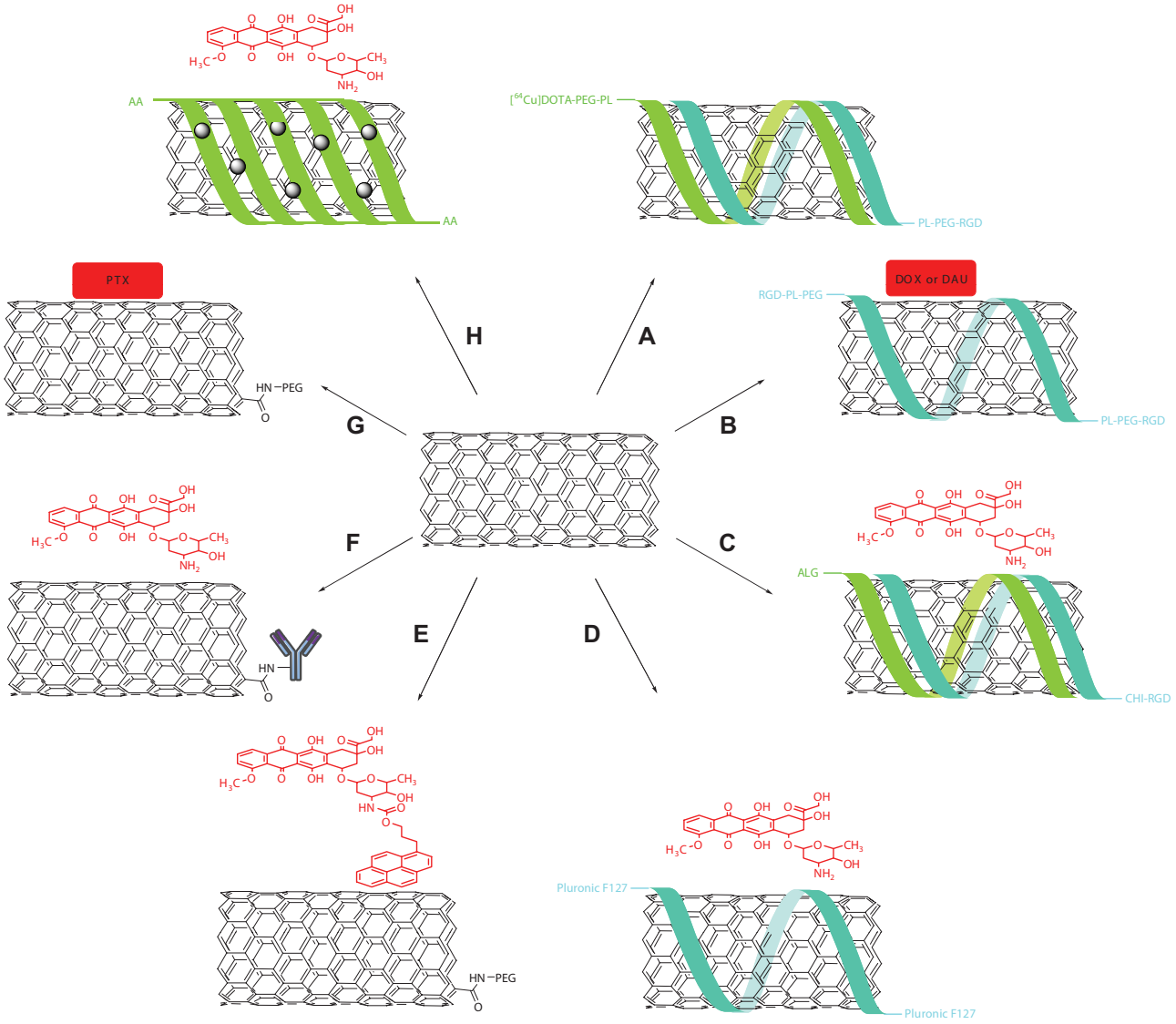

Figure 3 Different approaches for the non-covalent double functionalization of CNTs.

π-π stacking of its aromatic chromophore groups and the CNT backbone. The *in vitro* cytotoxicity studies on human breast cancer cells showed that the MWCNT-DOX complex exhibited enhanced cytotoxic capacity compared to both DOX alone and the DOX-pluronic complex.

In contrast to direct attachments of DOX onto the nanotube surface, Sengupta and coworkers [55] linked DOX by a carbamate bond to pyrene groups and the latter adsorbed by π-π stacking on the nanotube sidewall (Figure 3, Approach E). The carbamate linker was chosen for achieving a controlled enzymatic cleavage of DOX in cancer cell lysates [56]. This novel CNT-DOX formulation was used in a mouse melanoma tumor model demonstrating similar reduction in tumor volume than free DOX but with the advantage of non-toxic side effects, as evidenced by the unchanged body weights of the mice.

Recently, Li et al. [57] proposed for the first time SWCNTs functionalized with the antibody anti P-glycoprotein (P-gp) for targeting the multidrug resistant (MDR) human leukemia cells. Indeed, multidrug resistance is the major obstacle for successful cancer chemotherapy and it is mainly caused by membrane P-gp overexpression in the multidrug resistant cells that increases the out-efflux of anticancer drugs [58]. To overcome this problem, DOX was loaded onto anti-P-gp-SWCNTs (Ap-SWCNTs) via non-covalent physical adsorption to selectively eliminate the MDR cells (Figure 3, Approach F). The obtained maximum loading capacity for Ap-SWCNTs was approximately 40 μg/mg of DOX. The *in vitro* drug release from the nanotubes into the target cells was controlled via exposure to near-infrared (NIR) radiation. In fact, it is known that CNTs absorb energy from NIR light, [59] and this process induces the drug release due to endothermic desorption of the molecules from the nanotube surface. The targeting ability of Ap-SWCNT-DOX towards the membrane P-gp of the human leukemia cells (K562R) together with the intracellular uptake were confirmed by flow cytometry and confocal laser scanning microscopy. Finally, the Ap-SWCNT-

DOX conjugates expressed 2.4-fold higher cytotoxicity than free DOX in cell viability tests, suggesting that Ap-SWCNTs are promising vehicles for targeted drug delivery to suppress the proliferation of MDR cells for cancer therapy.

Lay et al. [60] extended the use of pegylated CNTs as delivery systems of paclitaxel (PTX) [61], a potent chemotherapeutic drug. Oxidized SWCNTs and MWCNTs were covalently functionalized via amidation with PEG segments and then loaded with PTX (Figure 3, Approach G) in methanol due to its very low solubility in aqueous solution. The PTX loading onto CNTs, based on strong hydrophobic interactions, improved the water solubility of PTX. Indeed, the PTX-loaded CNT-PEG was well dispersed in aqueous solutions without apparent evidence of aggregation. Moreover, the PTX-CNT-PEG conjugates exhibited high efficacy to kill MCF-7 breast and HeLa cervical cancer cells with IC_{50} lower than free PTX, confirming the increased bioavailability and cell-penetrating ability of PTX due to pegylated-CNT carriers.

In another bifunctional approach, Guo et al. [62] developed MWCNTs for *in vivo* imaging and drug delivery in the early detection and treatment of cancer. In their study, the nanotube surface was modified by plasma polymerization to deposit ultra-thin acrylic acid (AA) or poly(lactic-*co*-glycolic acid) (PLGA) films (3–6 nm) [63]. The AA-coating film promoted and facilitated the conjugation of CdSe/ZnS QDs [64] with the nanotube surface (Figure 3, Approach H). The CNTs coupled with QDs displayed a suitable NIR emission at 752 nm, allowing the non-invasive imaging of live mice to determine the biodistribution of CNT-QD in the various organs after injection. Finally, the high capacity of the polymer-coated MWCNTs to non-covalently adsorb PTX was also demonstrated, and the resulting PTX-loaded nanotubes exhibited *in vitro* antitumor efficacy against PC-3MM2 human prostate cancer cells. These data point out the great potential of CNT-QD conjugates not only as drug delivery systems but also as non-invasive optical *in vivo* imaging platforms.

In 2008, Zhang et al. [65] reported the double functionalization of carbon nanohorns using a non-covalent/covalent approach. Oxidized single-walled CNHs (SWNHs) with opened holes were first non-covalently loaded with zinc phthalocyanine (ZnPc) molecules via π-π stacking and then covalently derivatized via amidation with the protein bovine serum albumin (BSA) to make the resulting system hydrophilic and biocompatible [66]. The ZnPc-SWNH-BSA construct combines the photothermal or photohyperthermia (PHT) properties of CNHs with the photodynamic therapy (PDT) [67] effect of ZnPc for applications in double cancer phototherapy. Although the ZnPc or SWNH-BSA treatment alone had some *in vivo* antitumor effects, the ZnPc-SWNH-BSA treatment using a single laser-irradiation showed the disappearance of the tumor, demonstrating the importance of the PHT/PDT double effect in cancer therapy. The latter is only possible with CNHs because they have PHT properties by themselves and additionally can be loaded with PDT agents. Moreover, the *in vivo* toxicological and biodistribution assessments of CNHs [68] seem to encourage the design of this nanomaterial for biomedical applications.

All these studies emphasize once more the versatility of the multifunctionalization of carbon nanomaterials as platforms for the adsorption or the attachment of drugs, targeting and imaging molecules for therapeutic and diagnostic purposes against cancer diseases and other pathologies.

4. Triple functionalization of carbon nanotubes

The high aspect ratio and specific surface area of CNTs allow preparing even more highly advanced nanomaterials. The possibility to functionalize CNTs with more than two molecules is feasible and it opens interesting opportunities for the use of CNTs as multimodal nanomaterials by imparting magnetic susceptibility, imaging signals, targeting capability and/or drug loading capacity. In this context, few strategies for the covalent, non-covalent or mixed triple functionalization of CNTs have been reported so far. Ruggerio et al. prepared SWCNTs functionalized with three different tracking probes, corresponding to two fluorescent dyes and a metal-ion chelating agent, for *in vivo* multimodal imaging of the nanotubes. The design of these conjugates was aimed to investigate the clearance route (Figure 4, Approach A) [69]. For this purpose, SWCNTs were first functionalized by 1,3-dipolar cycloaddition of azomethine ylides to introduce pyrrolidine rings on the nanotube surface bearing amino groups on the lateral chain. The amine loading was determined using a colorimetric assay. The Alexa Fluor (AF) 488 dye was then reacted in defect amount to the amines. A second dye, Alexa Fluor 680, was then grafted on the amino groups again in defect. After each coupling, the amount of the fluorescent probes was determined by UV/Vis spectroscopy. Finally, the remaining amino functions of the dual-fluorescently labeled SWCNTs were reacted with the bifunctional chelating agent DOTA and subsequently radiolabeled with ^{86}Y. The functionalization of SWCNTs with the three imaging probes allowed to track the nanotubes in mice by using dynamic PET, NIR fluorescence imaging of the kidney, as well as immunohistochemistry and immune fluorescence imaging of the nephron. This multimodal tracking approach allowed assessing a rapid and effective CNT elimination from the blood circulation by the kidney without concomitant degradation.

Recently, our group described a simple, efficient and well-controlled approach based on a one-pot process for the covalent functionalization of CNTs with three different functional groups [70]. Our strategy relies on the simultaneous reaction of a mixture of three aryldiazonium salts generated *in situ*, leading to arylation of the nanotube surface (Figure 4, Approach B). The CNTs were functionalized with benzylamine moieties blocked with three quasi-orthogonal protecting groups that could be selectively removed under specific conditions. The sequential removal of the protecting groups of the amine functions allows the grafting of molecules of interest, such as a drug, a targeting ligand and a tracking probe, onto CNTs in a sequential and controlled manner. The triple functionalized CNTs were fully characterized by spectroscopy and microscopy techniques and the level of functionalization was assessed by using a colorimetric test that determines the amount of primary amine functions.

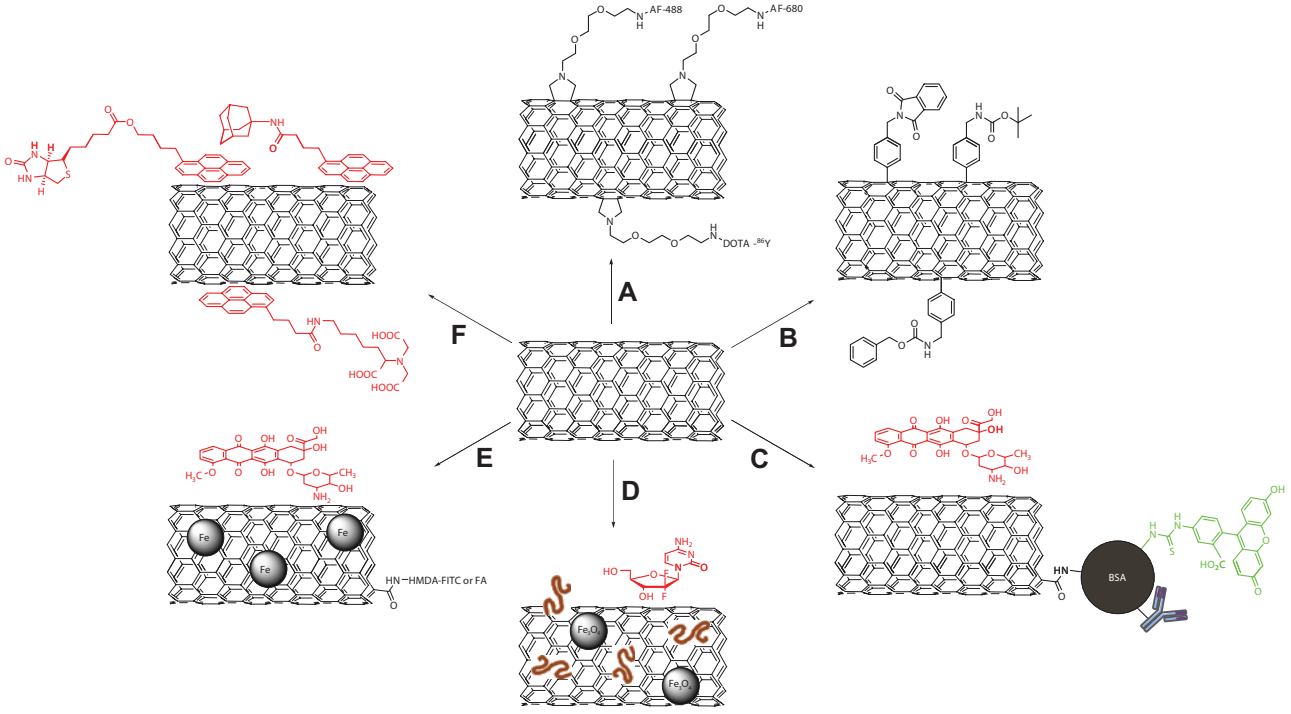

Figure 4 Different approaches for the triple functionalization of CNTs.

In a study reported by the McFadden group, a combination of covalent and non-covalent binding was used to functionalize SWCNTs with three different molecules of interest: (i) the anticancer drug DOX, (ii) a monoclonal antibody for molecular targeting, and (iii) a fluorescent probe for imaging (Figure 4, Approach C) [71]. The selected antibody recognizes a carcinoembryonic antigen which is a tumor marker for the identification of metastatic diseases. SWCNTs were labeled with fluorescein to allow the colocalization of CNTs and fluorescent DOX inside the cells. BSA was used to covalently link both the antibody and fluorescein to SWCNTs as it contains 60 amino groups from the lysine side chains and 99 carboxylic groups from the glutamic and aspartic acid residues. First, some amino functions of BSA were derivatized with fluorescein and the remaining amino groups were then coupled to COOH functions of oxidized SWCNTs which were previously loaded with DOX by adsorption on the nanotube sidewall. Finally, the antibody was coupled to the carboxylic functions of BSA. This approach is original, but rather complex, and it suffers from a thorough characterization and precise determination of the levels of functionalization. The uptake experiments showed that the CNT conjugates were internalized into cancer cells with subsequent intracellular release of DOX, probably triggered by the lower pH inside endosomes. The drug then translocated to the nucleus to exert its cytotoxic action, while the nanotubes remained in the cytoplasm.

By combining covalent and non-covalent functionalization, Yang et al. reported the decoration of MWCNTs with magnetite nanoparticles and subsequent loading with an anticancer drug for application as lymphatic targeted drug delivery systems (Figure 4, Approach D) [72]. Grafting polymerization of acrylonitrile was performed in micelles of MWCNTs obtained by dispersion with sodium dodecylbenzene sulfonate. After hydrolysis of polyacrylonitrile moieties, polyacrylic acid (PAA)-functionalized MWCNTs were obtained with a PAA grafting ratio of 15% in weight. Chemical co-precipitation of Fe^{2+} and Fe^{3+} salts onto the surface of PAA-functionalized MWCNTs dispersed in water led to a uniform coating of Fe_3O_4 nanoparticles, providing high magnetic susceptibility to the MWCNT hybrids. The cancer chemotherapy drug gemcitabine was then loaded by physical adsorption on the doubly functionalized MWCNTs. After subcutaneous administration in rats, the functionalized MWCNTs were taken up into lymph vessels under the guidance of a magnetic field. Gemcitabine was delivered with high efficiency into lymphatic nodes of the rats due to the enhanced permeation retention (EPR) effect. This study highlights the possibility to target CNTs to specific areas owing to the combination with magnetic nanoparticles.

In another study, MWCNTs were rendered magnetic by decoration with metallic iron nanoparticles. They were also functionalized with DOX and folate or fluorescein to exploit the CNT-based nanohybrids as dual-targeted drug nanocarriers to cancer cells [73]. To this aim, the MWCNTs were oxidized and Fe^{3+} salts were adsorbed on the nanotube surface and subsequently transformed to iron nanoparticles of 5–7 nm in diameter by heat treatment and reduction using hydrogen. The carboxyl functions of oxidized MWCNTs loaded with iron nanoparticles were then derivatized with folic acid conjugated with hexamethylenediamine (HMDA) and/or FITC conjugated to HMDA. Finally, DOX was adsorbed on the nanotube surface by π-stacking (Figure 4, Approach E). Owing to the presence of both folic acid and the magnetic iron nanoparticles, the MWCNT conjugates were guided to the location of cancer cells by applying an external magnetic field. The release of

DOX into HeLa cells was improved by NIR exposure via a thermal process. Taken together, these data demonstrate that the use of both biologically active and magnetically passive targeting allows to increase the delivery efficiency of DOX *in vitro* by 6-fold in comparison to the free drug.

Finally, an original approach for the non-covalent triple functionalization of SWCNTs was proposed by Holzinger et al. and applied to the development of biosensors [74]. Three different pyrene derivatives were simultaneously immobilized on the nanotube surface by π-stacking in a one-step reaction by simple dip coating (Figure 4, Approach F). This procedure was performed on nanotube-coated electrodes for the elaboration of multivalent biosensors. Adamantane-pyrene, biotin-pyrene and nitrilotriacetic acid (NTA)-pyrene were adsorbed on the nanotube sidewall to allow the step-by-step immobilization of β-cyclodextrin modified glucose oxidase (GOX) via supramolecular host-guest interactions with the adamantane groups, biotinylated GOX via avidin bridges, and histidine modified GOX via coordination with the NTA-Cu^{2+} complex. The presence of the functional groups was assessed by amperometry after subsequent immobilization of the biomolecules. This method is fast and reproducible and can also be extended to prepare new multifunctional composite material.

Overall, the different methods developed for the triple functionalization of CNTs allow imparting a combination of properties to the nanotubes, which is particularly needed for the use of functionalized CNTs in nanomedicine.

5. Summary and future directions

The increasing importance of nanotechnology in the biomedical field and the recent progress of nanoscience and biomedicine have spurred the development of highly advanced nanoscale drug delivery systems with multiple functions and complex capabilities. The main advantage of nanocarriers resides in the possibility to impart multifunctionality. Indeed, multimodal nanosystems with targeting, imaging and drug delivery capabilities within a single nanoscale construct hold tremendous promise in the treatment of different types of diseases and in particular for cancer therapy. Theragnosis, which combines simultaneous diagnostics and therapeutics, represents a new modality that utilizes dual therapy and real-time non-invasive *in vivo* imaging. This specific combination of functions can provide biodistribution information, opportunities to study therapeutic mechanisms, and strategies for improving therapeutic efficacy and reducing side effects.

In this review, we have emphasized the unique properties of CNTs that allow them to be used as multimodal nanomaterials. Owing to high specific surface area of CNTs, multiple copies of different molecules can be introduced on the nanotube surface, which opens the door to perform multiple functions. The sidewall of CNTs can be modified *via* adsorption or covalent bonding to improve biocompatibility, achieve passive or targeted delivery to tumors, deliver drugs and allow imaging. Compounds can also be encapsulated within the interior core of CNTs. Few studies reported the loading of the internal cavity of CNTs with drugs or imaging contrast agents. In addition to imaging signals, targeting capability, and/or drug loading capacity, magnetic susceptibility can be imparted to the CNT conjugates through decoration of their sidewall with metallic iron or Fe_3O_4 nanoparticles. These nanoparticles can serve not only as magnetic resonance imaging contrast agents for *in vivo* imaging but also as vehicles for manipulation by magnetic fields to improve drug delivery or to allow for localized heat therapy.

Even more sophisticated CNT-based nanovectors could be designed by exploiting the unique electrical properties of SWCNTs and their potential as *in vivo* or *ex vivo* sensors. In this context, a novel functionality could be brought to the multimodal CNT system by detecting targets inside the body and adapting their function accordingly. In addition, the exceptional and versatile physicochemical properties of SWCNTs offer great opportunities for a variety of imaging modalities such as radionuclide-based imaging, magnetic resonance, NIR fluorescence, Raman spectroscopy and photoacoustic tomography. SWCNT-based nanosensors and *in vivo* imaging are important for optimization of disease diagnosis and patient management in the future. Furthermore, the ability of CNTs to absorb NIR light and convert it into heat affords opportunities for cancer phototherapy and combined treatments. Hyperthermia has been clinically used in the treatment of solid tumors because it can enhance the efficiency of chemo- or radiotherapy. SWCNTs are also able to generate heat in a radiofrequency (RF) field, which can be applied to ablate tumors as the RF field has excellent tissue penetration ability.

In summary, the unique properties of CNTs may profoundly impact disease diagnosis and treatment and they allow for a series of novel cancer therapies such as photothermal therapy, radiofrequency ablation treatment of tumors, as well as photoacoustic therapy. Upon further development, approaches relying on multifunctional CNT-based nanosystems will offer new possibilities towards the development of personalized medicine, by predicting which patients will probably respond to a specific molecular therapy and monitor their response accordingly. The possibility of treating each patient based on individual profile provides challenging opportunities in nanomedicine.

Current scientific activities of the authors

The group entitled “Carbon-based nanomaterials and delivery”, led by Alberto Bianco, belongs to UPR 9021 “Immunologie et Chimie Thérapeutiques” of the French National Research Council (CNRS) located in Strasbourg (France). The group is mainly composed of organic chemists interested in developing new functional nanomaterials. In particular, the research activity of our team focuses on the development of novel vectors based on carbon nanomaterials (carbon nanotubes, fullerene and adamantane) for biomedical applications. The covalent or non-covalent immobilization of biologically active molecules on the external surface of carbon nanotubes has permitted to engineer biomolecular complexes and novel conjugates that we have exploited for different applications including drug delivery, gene transfer

and RNA silencing. For this purpose, we have devised different strategies to functionalize the external surface and the terminal parts of the CNTs using the cycloaddition and amidation reactions, generating highly dispersible carbon-based materials. We are thoroughly studying the toxicity effects of functionalized CNTs and analyzing the mechanisms of cell penetration, organ biodistribution and the routes of elimination following *in vivo* administration. We are exploiting the positive charges of ammonium functionalized CNTs for the formation of complexes with short interference RNA as these conjugates have a great potential for *in vivo* gene silencing. We are also exploring the multifunctionalization of CNTs to link different types of molecules. We have created a system not only capable of delivering a therapeutic agent (i.e., amphotericin B, methotrexate and doxorubicin) but that can be tracked because of its fluorescence. Moreover, this system is capable of tackling the desired organ or tissue because modified with a targeting ligand. Overall, the obtained results aim to develop functionalized CNTs as an emergent new class of carriers in drug discovery and delivery. Very recently, we started to develop a new system based on a muntifunctionalized dendritic structure, containing adamantane as the main core, for therapeutic, imaging and diagnostic applications in nanomedicine.

References

[1] Iijima S. Helical microtubules of graphitic carbon. *Nature* 1991, 354, 56–58.

[2] Salvetat-Delmotte J-P, Rubio A. Mechanical properties of carbon nanotubes: a fiber digest for beginners. *Carbon* 2002, 40, 1729–1734.

[3] Avouris P. Molecular electronics with carbon nanotubes. *Acc. Chem. Res.* 2002, 35, 1026–1034.

[4] Schnorr JM, Swager TM. Emerging applications of carbon nanotubes. *Chem. Mater.* 2011, 23, 646–657.

[5] Byrne MT, Gun'ko YK. Recent advances in research on carbon nanotube-polymer composites. *Adv. Mater.* 2010, 22, 1672–1688.

[6] Kostarelos K, Bianco A, Prato M. Promises, facts and challenges for carbon nanotubes in imaging and therapeutics. *Nat. Nanotechnol.* 2009, 4, 627–633.

[7] a) Karousis N, Tagmatarchis N, Tasis D. Current progress on the chemical modification of carbon nanotubes. *Chem. Rev.* 2010, 110, 5366–5397; b) Singh P, Campidelli S, Giordani S, Bonifazi D, Bianco A, Prato M. Organic functionalisation and characterisation of single-walled carbon nanotubes. *Chem. Soc. Rev.* 2009, 38, 2214–2230.

[8] Liu J, Rinzler AG, Dai H, Hafner JH, Bradley RK, Boul PJ, Lu A, Iverson T, Shelimov K, Huffman CB, Rodriguez-Macias F, Shon YS, Lee TR, Colbert DT, Smalley RE. Fullerene pipes. *Science* 1998, 280, 1253–1256.

[9] Bahr JL, Yang J, Kosynkin DV, Bronikowski MJ, Smalley RE, Tour JM. Functionalization of carbon nanotubes by electrochemical reduction of aryl diazonium salts: a bucky paper electrode. *J. Am. Chem. Soc.* 2001, 123, 6536–6542.

[10] a) Tagmatarchis N, Prato M. Functionalization of carbon nanotubes via 1,3-dipolar cycloadditions. *J. Mater. Chem.* 2004, 14, 437–439; b) Rana S, Cho JW. Functionalization of carbon nanotubes via Cu(I)-catalyzed Huisgen [3+2] cycloaddition "click chemistry". *Nanoscale* 2010, 2, 2550–2556.

[11] Zhao Y-L, Stoddart JF. Noncovalent functionalization of single-walled carbon nanotubes. *Acc. Chem. Res.* 2009, 42, 1161–1171.

[12] Debnath S, Cheng Q, Hedderman TG, Byrne HJ. A study of the interaction between single-walled carbon nanotubes and polycyclic aromatic hydrocarbons: toward structure-property relationships. *J. Phys. Chem. C* 2008, 112, 10418–10422.

[13] Angelikopoulos P, Gromov A, Leen A, Nerushev O, Bock H, Campbell EEB. Dispersing individual single-wall carbon nanotubes in aqueous surfactant solutions below the cmc. *J. Phys. Chem. C* 2010, 114, 2–9.

[14] Gao J, Loi MA, Figueiredo de Carvalho EJ, dos Santos MC. Selective wrapping and supramolecular structures of polyfluorene-carbon nanotube hybrids. *ACS Nano* 2011, 5, 3993–3999.

[15] Barone PW, Baik S, Heller DA, Strano MS. Near-infrared optical sensors based on single-walled carbon nanotubes. *Nat. Mater.* 2005, 4, 86–92.

[16] Lu F, Gu L, Meziani MJ, Wang X, Luo PG, Veca LM, Cao L, Sun YP. Advances in bioapplications of carbon nanotubes. *Adv. Mater.* 2009, 21, 139–152.

[17] Gaillard C, Cellot G, Li S, Toma FM, Dumortier H, Spalluto G, Cacciari B, Prato M, Ballerini L, Bianco A. Carbon nanotubes carrying cell-adhesion peptides do not interfere with neuronal functionality. *Adv. Mater.* 2009, 21, 2903–2908.

[18] Chen RJ, Zhang Y, Wang D, Dai H. Noncovalent sidewall functionalization of single-walled carbon nanotubes for protein immobilization. *J. Am. Chem. Soc.* 2001, 123, 3838–3839.

[19] Lacerda L, Bianco A, Prato M, Kostarelos K. Carbon nanotube cell translocation and delivery of nucleic acids in vitro and in vivo. *J. Mater. Chem.* 2008, 18, 17–22.

[20] a) Liu Z, Chen K, Davis C, Sherlock S, Cao Q, Chen X, Dai H. Drug delivery with carbon nanotubes for in vivo cancer treatment. *Cancer Res.* 2008, 68, 6652–6660; b) Dhar S, Liu Z, Thomale J, Dai H, Lippard SJ. Targeted single-wall carbon nanotube-mediated Pt(IV) prodrug delivery using folate as a homing device. *J. Am. Chem. Soc.* 2008, 130, 11467–11476.

[21] Ménard-Moyon C, Venturelli E, Fabbro C, Samorí C, Da Ros T, Kostarelos K, Prato M, Bianco A. The alluring potential of functionalized carbon nanotubes in drug discovery. *Expert Opin. Drug Discovery* 2010, 5, 691–707.

[22] Ménard-Moyon C, Kostarelos K, Prato M, Bianco A. Functionalized carbon nanotubes for probing and modulating molecular functions. *Chem. Biol.* 2010, 17, 107–115.

[23] a) Pantarotto D, Singh R, McCarthy D, Erhardt M, Briand J-P, Prato M, Kostarelos K, Bianco A. Functionalized carbon nanotubes for plasmid DNA gene delivery. *Angew. Chem. Int. Ed.* 2004, 43, 5242–5246; b) Cheung W, Pontoriero F, Taratula O, Chen AM, He H. DNA and carbon nanotubes as medicine. *Adv. Drug Delivery Rev.* 2010, 62, 633–649.

[24] a) Podesta JE, Al-Jamal KT, Herrero MA, Tian B, Ali-Boucetta H, Hegde V, Bianco A, Prato M, Kostarelos K. Antitumor activity and prolonged survival by carbon-nanotube-mediated therapeutic siRNA silencing in a human lung xenograft model. *Small* 2009, 5, 1176–1185; b) 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, 2023–2027.

[25] Singhal R, Orynbayeva Z, Kalyana S, Ramalingam V, Niu JJ, Bhattacharyya S, Vitol EA, Schrlau MG, Papazoglou ES, Friedman G, Gogotsi Y. Multifunctional carbon-nanotube cellular endoscopes. *Nature Nanotechnol.* 2011, 6, 57–64.

[26] Hong H, Gao T, Cai W. Molecular imaging with single-walled carbon nanotubes. *Nano Today* 2009, 4, 252–261.

[27] Lee KM, Lingchuan L, Dai L. Asymmetric end-functionalization of multi-walled carbon nanotubes. *J. Am. Chem. Soc.* 2005, 127, 4122–4123.

[28] Moghaddam MJ, Taylor S, Gao M, Huang S, Dai L, McCall MJ. Highly efficient binding of DNA on the sidewalls and tips of carbon nanotubes using photochemistry. *Nano Lett.* 2004, 4, 89–93.

[29] Holzinger M, Vostrowsky O, Hirsch A, Hennrich F, Kappes M, Weiss R, Jellen F. Sidewall functionalization of carbon nanotubes. *Angew. Chem. Int. Ed.* 2001, 40, 4002–4005.

[30] Wu W, Wieckowski S, Pastorin G, Benincasa M, Klumpp C, Briand J-P, Gennaro R, Prato M, Bianco A. Targeted delivery of amphotericin B to cells by using functionalized carbon nanotubes. *Angew. Chem. Int. Ed.* 2005, 44, 6358–6362.

[31] Benincasa M, Pacor S, Wu W, Prato M, Bianco A, Gennaro R. Antifungal activity of amphotericin B conjugated to carbon nanotubes. *ACS Nano* 2011, 5, 199–208.

[32] Chen J, Chen S, Zhao X, Kuznetsova LV, Wong SS, Ojima I. Functionalized single-walled carbon nanotubes as rationally designed vehicles for tumor-targeted drug delivery. *J. Am. Chem. Soc.* 2008, 130, 16778–16785.

[33] Villa CH, McDevitt MR, Escorcia FE, Rey DA, Bergkvist M, Batt CA, Scheinberg DA. Synthesis and biodistribution of oligonucleotide-functionalized, tumor-targetable carbon nanotubes. *Nano Lett.* 2008, 8, 4221–4228.

[34] Samorí C, Ali-Boucetta H, Sainz R, Guo C, Toma FM, Fabbro C, da Ros T, Prato M, Kostarelos K, Bianco A. Enhanced anticancer activity of multi-walled carbon nanotube-methotrexate conjugates using cleavable linkers. *Chem. Commun.* 2010, 46, 1494–1496.

[35] Pastorin G, Wu W, Wieckowski S, Briand J-P, Kostarelos K, Prato M, Bianco A. Double functionalisation of carbon nanotubes for multimodal drug delivery. *Chem. Commun.* 2006, 1182–1184.

[36] McDevitt MR, Chattopadhyay D, Kappel BJ, Jaggi JS, Schiffman SR, Antczak C, Njardarson JT, Brentjens R, Scheinberg DA. Tumor targeting with antibody-functionalized, radiolabeled carbon nanotubes. *J. Nucl. Med.* 2007, 48, 1180–1189.

[37] Ruggiero A, Villa CH, Holland JP, Sprinkle SR, May C, Lewis JS, Scheinberg DA, McDevitt MR. Imaging and treating tumor vasculature with targeted radiolabeled carbon nanotubes. *Int. J. Nanomed.* 2010, 5, 783–802.

[38] Bhirde AA, Patel V, Gavard J, Zhang G, Sousa AA, Masedunskas A, Leapman RD, Weigert R, Gutkind JS, Rusling JF. Targeted killing of cancer cells in vivo and in vitro with EGF-directed carbon nanotube-based drug delivery. *ACS Nano* 2009, 3, 307–316.

[39] Wu W, Li R, Bian X, Zhu Z, Ding D, Li X, Jia Z, Jiang X, Hu Y. Covalently combining carbon nanotubes with anticancer agent: preparation and antitumor activity. *ACS Nano* 2009, 3, 2740–2750.

[40] Brunetti FG, Herrero MA, Muñoz J de M, Díaz-Ortiz A, Alfonsi J, Meneghetti M, Prato M, Vázquez E. Microwave-induced multiple functionalization of carbon nanotubes. *J. Am. Chem. Soc.* 2008, 130, 8094–8100.

[41] Rubio N, Herrero MA, de la Hoz A, Meneghetti M, Prato M, Vázquez E. Versatile microwave-induced reactions for the multiple functionalization of carbon nanotubes. *Org. Biomol. Chem.* 2010, 8, 1936–1942.

[42] Brunetti FG, Herrero MA, Muñoz J de M, Giordani S, Díaz-Ortiz A, Filippone S, Ruaro G, Meneghetti M, Prato M, Vázquez E. Reversible microwave-assisted cycloaddition of aziridines to carbon nanotubes. *J. Am. Chem. Soc.* 2007, 129, 14580–14581.

[43] Liu J, Zubiri MR, Vigolo B, Dossot M, Humbert B, Fort Y, McRae E. Microwave-assisted functionalization of single-wall carbon nanotubes through diazonium. *J. Nanosci. Nanotechnol.* 2007, 7, 3519–3523.

[44] Iijima S, Yudasaka M, Yamada R, Bandow S, Suenaga K, Kokai F, Takahashi K. Nano-aggregates of single-walled graphitic carbon nano-horns. *Chem. Phys. Lett.* 1999, 309, 165–170.

[45] Rubio N, Herrero MA, Meneghetti M, Díaz-Ortiz Á, Schiavon M, Prato M, Vázquez E. Efficient functionalization of carbon nanohorns via microwave irradiation. *J. Mater. Chem.* 2009, 19, 4407–4413.

[46] Stephenson JJ, Hudson JL, Leonard AD, Price BK, Tour JM. Repetitive functionalization of water-soluble single-walled carbon nanotubes. Addition of acid-sensitive addends. *Chem. Mater.* 2007, 19, 3491–3498.

[47] Liu Z, Cai W, He L, Nakayama N, Chen K, Sun X, Chen X, Dai H. In vivo biodistribution and highly efficient tumour targeting of carbon nanotubes in mice. *Nat. Nanotechnol.* 2007, 2, 47–52.

[48] a) Jin H, Varner J. Integrins: roles in cancer development and as treatment targets. *Br. J. Cancer* 2004, 90, 561–565; b) Xiong J-P, Stehle T, Zhang R, Joachimiak A, Frech M, Goodman SL, Arnaout MA. Crystal structure of the extracellular segment of integrin $\alpha_v\beta_3$ in complex with an Arg-Gly-Asp ligand. *Science* 2002, 296, 151–155.

[49] Liu Z, Sun X, Nakayama-Ratchford N, Dai H. Supramolecular chemistry on water-soluble carbon nanotubes for drug loading and delivery. *ACS Nano* 2007, 1, 50–56.

[50] Liu Z, Fan AC, Rakhra K, Sherlock S, Goodwin A, Chen X, Yang Q, Felsher DW, Dai H. Supramolecular stacking of doxorubicin on carbon nanotubes for in vivo cancer therapy. *Angew. Chem. Int. Ed.* 2009, 48, 7668–7672.

[51] Gabizon A, Shmeeda H, Barenholz Y. Pharmacokinetics of pegylated liposomal doxorubicin: review of animal and human studies. *Clin. Pharmacokinet.* 2003, 42, 419–436.

[52] Zhang X, Meng L, Lu Q, Fei Z, Dyson PJ. Targeted delivery and controlled release of doxorubicin to cancer cells using modified single wall carbon nanotubes. *Biomaterials* 2009, 30, 6041–6047.

[53] Sudimack J, Lee RJ. Targeted drug delivery via the folate receptor. *Adv. Drug Deliv. Rev.* 2000, 41, 147–162.

[54] Ali-Boucetta H, Al-Jamal KT, McCarthy D, Prato M, Bianco A, Kostarelos K. Multiwalled carbon nanotube-doxorubicin supramolecular complexes for cancer therapeutics. *Chem. Commun.* 2008, 459–461.

[55] Chaudhuri P, Soni S, Sengupta S. Single-walled carbon nanotube-conjugated chemotherapy exhibits increased therapeutic index in melanoma. *Nanotechnology* 2010, 21, 025102.

[56] 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, 2605–2611.

[57] Li R, Wu R, Zhao L, Wu M, Yang L, Zou H. P-glycoprotein antibody functionalized carbon nanotube overcomes the multidrug resistance of human leukemia cells. *ACS Nano* 2010, 4, 1399–1408.

[58] a) Clarke R, Leonessa F, Trock B. Multidrug resistance/P-glycoprotein and breast cancer: review and meta-analysis. *Semin. Oncol.* 2005, 32, 9–15; b) Zgurskaya HI, Nikaido H. Multidrug resistance mechanisms: drug efflux across two membranes. *Mol. Microbiol.* 2000, 37, 219–225; c) Li X, Li J-P, Yuan H-Y, Gao X, Qu X-J, Xu W-F, Tang W. Recent advances in P-glycoprotein-mediated multidrug resistance reversal mechanisms. *Methods Find. Exp. Clin. Pharmacol.* 2007, 29, 607–618.

[59] Kam NWS, 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, 11600–11605.
[60] Lay CL, Liu HQ, Tan HR, Liu Y. Delivery of paclitaxel by physically loading onto poly(ethylene glycol) (PEG)-graft-carbon nanotubes for potent cancer therapeutics. *Nanotechnology* 2010, 21, 065101.
[61] Rowinsky EK, Donehower RC. Paclitaxel (taxol). *New Engl. J. Med.* 1995, 332, 1004–1014.
[62] Guo Y, Shi D, Cho H, Dong Z, Kulkarni A, Pauletti GM, Wang W, Lian J, Liu W, Ren L, Zhang Q, Liu G, Huth C, Wang L, Ewing RC. In vivo imaging and drug storage by quantum-dot-conjugated carbon nanotubes. *Adv. Funct. Mater.* 2008, 18, 2489–2497.
[63] a) Shi D, Lian J, He P, Wang LM, van Ooij WJ, Schulz M, Liu Y, Mast DB. Plasma deposition of ultrathin polymer films on carbon nanotubes. *Appl. Phys. Lett.* 2002, 81, 5216–5218; b) Shi D, He P, Wang SX, van Ooij WJ, Wang LM, Zhao J, Yu Z. Interfacial particle bonding via an ultrathin polymer film on Al_2O_3 nanoparticles by plasma polymerization. *J. Mater. Res.* 2002, 17, 981–990; c) Shi D, Lian J, He P, Wang LM, Xiao F, Yang L, Schulz MJ, Mast DB. Plasma coating of carbon nanofibers for enhanced dispersion and interfacial bonding in polymer composites. *Appl. Phys. Lett.* 2003, 83, 5301–5303.
[64] Biju V, Itoh T, Anas A, Sujith A, Ishikawa M. Semiconductor quantum dots and metal nanoparticles: syntheses, optical properties, and biological applications. *Anal. Bioanal. Chem.* 2008, 391, 2469–2495.
[65] Zhang M, Murakami T, Ajima K, Tsuchida K, Sandanayaka ASD, Ito O, Iijima S, Yudasaka M. Fabrication of ZnPc/protein nanohorns for double photodynamic and hyperthermic cancer phototherapy. *Proc. Natl. Acad. Sci. USA* 2008, 105, 14773–14778.
[66] Zhang M, Yudasaka M, Ajima K, Miyawaki J, Iijima S. Light-assisted oxidation of single-wall carbon nanohorns for abundant creation of oxygenated groups that enable chemical modifications with proteins to enhance biocompatibility. *ACS Nano* 2007, 1, 265–272.
[67] a) Owens JW, Smith R, Robinson R, Robins M. Photophysical properties of porphyrins, phthalocyanines and benzochlorins. *Inorg. Chim. Acta* 1998, 279, 226–231; b) Chan W-S, Brasseur N, La Madeleine C, Ouellet R, van Lier JE. Efficacy and mechanism of aluminium phthalocyanine and its sulphonated derivatives mediated photodynamic therapy on murine tumours. *Eur. J. Cancer* 2001, 33, 1855–1859; c) Allen CM, Sharman WM, Van Lier JE. Current status of phthalocyanines in the photodynamic therapy of cancer. *J. Porphyr. Phthalocya.* 2001, 5, 161–169.
[68] a) Miyawaki J, Yudasaka M, Azami T, Kubo Y, Iijima S. Toxicity of single-walled carbon nanohorns. *ACS Nano* 2008, 2, 213–226; b) Tahara Y, Miyawaki J, Zhang M, Yang M, Waga I, Iijima S, Irie H, Yudasaka M. Histological assessments for toxicity and functionalization-dependent biodistribution of carbon nanohorns. *Nanotechnology* 2011, 22, 265106.
[69] Ruggiero A, Villa CH, Bander E, Rey DA, Bergkvist M, Batt CA, Manova-Todorova K, Deen WM, Scheinberg DA, McDevitt MR. Paradoxical glomerular filtration of carbon nanotubes. *Proc. Natl. Acad. Sci. USA* 2010, 107, 12369–12374.
[70] Ménard-Moyon C, Fabbro C, Prato M, Bianco A. One-pot triple functionalization of carbon nanotubes. *Chem. Eur. J.* 2011, 17, 3222–3227.
[71] Heister E, Neves V, Tîlmaciu CT, Lipert K, Beltrán VS, Coley HM, Ravi S, Silva P, McFadden J. Triple functionalisation of single-walled carbon nanotubes with doxorubicin, a monoclonal antibody, and a fluorescent marker for targeted cancer therapy. *Carbon* 2009, 47, 2152–2160.
[72] Yang D, Yang F, Hu J, Long J, Wang C, Fu D, Ni Q. Hydrophilic multi-walled carbon nanotubes decorated with magnetite nanoparticles as lymphatic targeted drug delivery vehicles. *Chem. Commun.* 2009, 4447–4449.
[73] Li R, Wua R, Zhao L, Hu Z, Guo S, Pan X, Zou H. Folate and iron difunctionalized multiwall carbon nanotubes as dual-targeted drug nanocarrier to cancer cells. *Carbon* 2011, 49, 1797–1805.
[74] Holzinger M, Baur J, Haddad R, Wang X, Cosnier S. Multiple functionalization of single-walled carbon nanotubes by dip coating. *Chem. Commun.* 2011, 47, 2450–2452.

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