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Functionalized Carbon Nanotubes in Drug Design and Discovery

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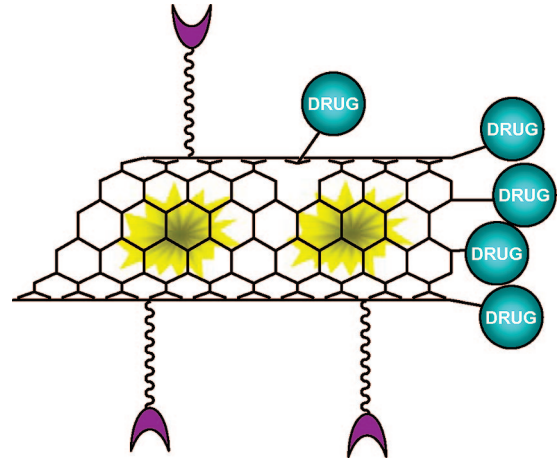
CONSPECTUS

Carbon nanotubes (CNTs) have been proposed and actively explored as multipurpose innovative carriers for drug delivery and diagnostic applications. Their versatile physicochemical features enable the covalent and noncovalent introduction of several pharmaceutically relevant entities and allow for rational design of novel candidate nanoscale constructs for drug development. CNTs can be functionalized with different functional groups to carry simultaneously several moieties for targeting, imaging, and therapy. Among the most interesting examples of such multimodal CNT constructs described in this Account is one carrying a fluorescein probe together with the antifungal drug amphotericin B or fluorescein and the antitumor agent methotrexate. The biological action of the drug in these cases is retained or, as in the case of amphotericin B constructs, enhanced, while CNTs are able to reduce the unwanted toxicity of the drug administered alone. Ammonium-functionalized CNTs can also be considered very promising vectors for gene-encoding nucleic acids. Indeed, we have formed stable complexes between cationic CNTs and plasmid DNA and demonstrated the enhancement of the gene therapeutic capacity in comparison to DNA alone. On the other hand, CNTs conjugated with antigenic peptides can be developed as a new and effective system for synthetic vaccine applications. What makes CNTs quite unique is their ability, first shown by our groups in 2004, to passively cross membranes of many different types of cells following a translocation mechanism that has been termed the *nanoneedle mechanism*. In that way, CNTs open innumerable possibilities for future drug discovery based on intracellular targets that have been hard to reach until today. Moreover, adequately functionalized CNTs as those shown in this Account can be rapidly eliminated from the body following systemic administration offering further encouragment for their development. CNT excretion rates and accumulation in organs and any reactivity with the immune system will determine the CNT safety profile and, consequently, any further pharmaceutical development. Caution is advised about the need for systematic data on the long-term fate of these very interesting and versatile nano-objects in correlation with the type of CNT material used. CNTs are gradually plyaing a bigger and more important role in the emerging field of nanomedicine; however, we need to guarantee that the great opportunities they offer will be translated into feasible and safe constructs to be included in drug discovery and development pipelines.



Introduction

The bioavailability and intrinsic toxicity of many potential low molecular weight drugs affect so much their pharmacological profile that their therapeutic efficacy is strongly compromised to the point that most of them have to be abandoned

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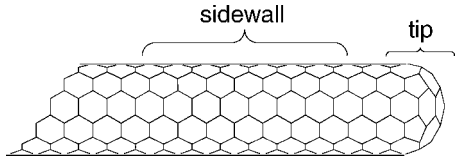
SCHEME 1. Molecular Structure of a Carbon Nanotube with Highlighted Tips and Sidewalls



from late-stage pharmaceutical development. This recurrent phenomenon in the drug development cycle has led to an almost critical situation plaguing the production pipelines of almost all pharmaceutical companies. This, in combination with the expiration of many patents protecting "blockbuster drugs", is currently forcing the pharmaceutical industry to act creatively in rejuvenating their drug discovery programs and design more and better drug candidates for clinical development. One of the possibilities that can act as a potential source of new drug design strategies is the incorporation of nanotechnologies at an early stage in the drug development process. Similar examples of alternative technologies (polymers, liposomes) into small molecule drug discovery can now be found in the market, including different types of drug products for a variety of indications, such as cancer and infectious diseases.[1,2] Several polymer- or lipid-based conjugate systems containing doxorubicin or amphotericin B have been shown to have increased efficacy and reduced toxicity. Moreover, these new drug conjugates are very expensive, and the search for new, more affordable constructs is highly desirable.[3]

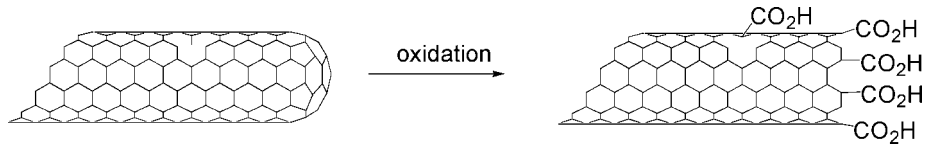
One of the most recent strategies proposed to incorporate nanotechnology principles to modulate the undesired effects of a drug and create new conjugates with promising and improved pharmacological profiles and modalities is through the application of carbon nanotube functionalization chemistry.[4] Besides the capacity of functionalized carbon nanotubes (*f*-CNT) to act as carriers for the delivery of a wide range of therapeutic agents, *f*-CNT conjugated to a drug can be also considered as a new entity with novel therapeutic or diagnostic properties.[5,6]

In this Account, we describe the efforts, mainly carried out in our laboratories, toward obtaining different types of fully functionalized CNT in which the carbon skeleton plays the fundamental role of a carrier with improved therapeutic efficacy mainly via multipresentation or multivalency.[7] In doing this, we propose a novel strategy for the development of new entities in drug design and discovery that can potentially constitute very attractive candidates for further pharmaceutical development.

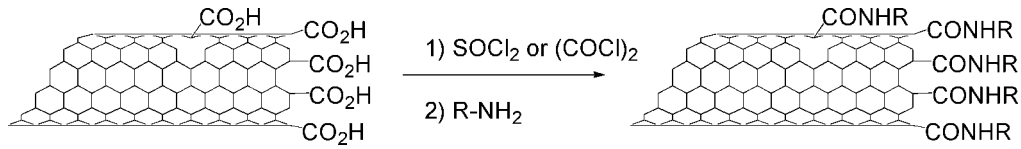
Carbon Nanotube Functionalization Chemistry

CNT are tubular objects with a high aspect ratio and a diameter in the nanoscale range.[8] They can be classified by their structure into two main types: (i) single-walled carbon nano-

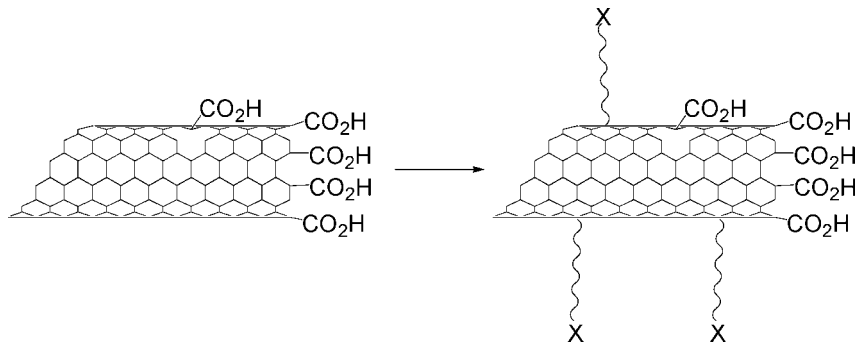
SCHEME 2. Oxidation of Carbon Nanotubes



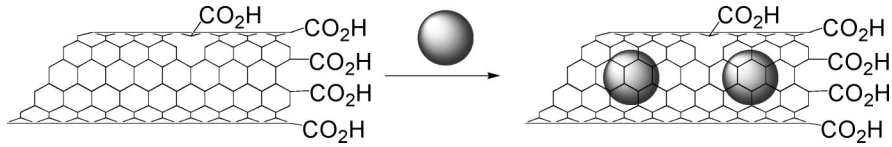
SCHEME 3. Amidation Reaction of Oxidized Carbon Nanotubes



SCHEME 4. Functionalization of Carbon Nanotubes Using Addition Reactions (X = Functional Groups)



SCHEME 5. Insertion inside Carbon Nanotubes



tubes (SWNT), which consist of a single layer of graphene sheet seamlessly rolled into a cylindrical tube,[9] and (ii) multiwalled carbon nanotubes (MWNT), which comprise multiple layers of concentric cylinders with the space of about 0.34 nm between the adjacent layers.[10]

From a chemical reactivity point of view, CNT can be differentiated in two zones: the *tips* and the *sidewalls* (Scheme 1).

The tips are reminiscent of the structure of a fullerene hemisphere and are relatively reactive.[4] The sidewalls can be approximately considered as curved graphite, the degree of curvature, of course, depending on the diameter of the tube.[11] However, whatever the diameter, the reactivity of the sidewalls is considerably lower than that of the tips. Therefore, most reactions are expected to occur at the tips first and then, in some cases, at the sidewalls, especially in the areas where defects are present (i.e., five- and seven-membered rings or holes due to an incomplete graphite arrangement). This difference in reactivity has led to a selective oxidation of the tips, while the sidewalls remain inert.

Since the as-produced CNT contain variable amounts of impurities, such as amorphous carbon and metallic nanoparticles, the initial efforts in their purification focused on the selective oxidation of the impurities with respect to the less reactive CNT. Use of strong oxidizing agents, such as concentrated nitric acid, led actually to purer materials.[12] However, since the CNT tips do generally react under these conditions, the result is that the tubes open and the tips consist now of oxygenated functions, mainly carboxylic acids.[13] Also, dangling bonds can react similarly, generating other functions at the sidewalls (Scheme 2).

The carboxylic functions can, in turn, lead to further derivatization.[14] After conversion to acid chlorides, reaction with amines can afford the corresponding amides (Scheme 3).

Another opportunity is given by the reactivity of the sidewalls. Cycloadditions or radical reactions can be employed to covalently attach molecular appendages to the CNT sidewalls[4] (Scheme 4).

Carbon Nanotubes as Nanocontainers

In principle, as the tips are open, CNT can also be considered as nanocontainers. Many molecules, ions, or metals can be possibly inserted.[15] A number of different molecules, such as fullerenes, porphyrins, and metals, have indeed been included in the internal space of CNT, mostly due to hydrophobic interactions.[16] Such constructs containing a metal or a metal complex can clearly constitute potential candidates for the design of pharmaceuticals for diagnostic purposes and will be developed as novel contrast agents for different imaging modalities (Scheme 5).

Functionalized Carbon Nanotubes for Therapeutic and Diagnostic Applications

If we assume that all the above-described processes can be efficiently performed, we will have reached the goal of implementing three different functional units for potential targeting, diagnosis, and drug transport capabilities. All new functional groups, including amines and carboxylates, can be further modified with therapeutic agents to create CNT conjugates endowed with some kind of pharmacological activity. Nanotubes able to carry one or more therapeutic moieties with optical or other (e.g., magnetic) probes for imaging, and/or specific recognition signals for targeting, can offer multimodal options in the treatment of cancer and other types of complex diseases where activity is required only at specific sites in the body. Of course, even when these synthetic objectives are reached, there will be several technological problems to solve, mainly of pharmaceutical development nature, among which are the stability of the complexes in physiological conditions, the degree of aggregation in vivo, the correct timing, and location of drug release. Nonetheless, considering the vast possibility of combinations offered, CNT are rich technological platforms for the development of candidates for simultaneous diagnosis, transport, and targeted delivery of drugs.[5,6]

The possibility of developing CNT for biomedical applications became a reality after a series of powerful methodologies for their functionalization were described.[4] Different approaches have been proposed to render the nanotubes soluble and compatible with physiological conditions. This is a fundamental issue for their integration into living system environments. A critical parameter to determine biocompatibility is the degree of toxicity of all CNT materials. This is a key issue which is currently under careful and extensive examination from various laboratories.[17] Observations reported by

other groups and ours so far have shown that functionalization remarkably reduces the cytotoxic effects of CNT,[18] while increasing their biocompatibility.[19] The evidence gathered so far highlights that the higher the degree of CNT functionalization, the safer is the material, particularly compared to pristine, purified CNT, thus offering the potential exploitation of nanotubes for drug administration.

Within the different types of organic reactions, we have developed the 1,3-dipolar cycloaddition reaction of azomethine ylides.[20,21] This method creates pyrrolidine rings distributed along the nanotube external walls and tips. We have also demonstrated that an oxidation/amidation followed by a cycloaddition reaction permits generation of doubly functionalized carbon nanotubes.[22]

The compounds shown in Table 1 can be synthesized along the lines described above. The main features of compounds **1**–**9** are as follows:

1. The presence of hydrophilic appendages. All of the compounds bear an aminotriethylene glycol chain for improving water solubility.

2. The presence of fluorescent probes as for compounds **3**, **4**, **5**, and **7**. Labeling can be performed, for instance, by condensing *f*-CNT **1** or **2** with fluorescein isothiocyanate (FITC), giving **3** and **4**, respectively. To improve water solubility, in some cases, e.g., **4**, it is possible to leave some of the amino groups unlabeled by playing with orthogonally protected functional groups.

3. The presence of amphotericin B as in the case of compounds **5** and **6**. Amphotericin B is a potent commercially available antifungal agent.

4. The presence of the anticancer agent methotrexate as in the case of compound **7**.

5. The presence of immunogenic peptides as in the case of compounds **8** and **9**.

All compounds in Table 1 were tested in various biological assays according to their structural properties (right column in Table 1).

In particular, compound **1** was tested in some of the first biocompatibility and cellular interaction studies ever carried out, whereby different types of human and murine cell lines, lymphocytes, and macrophages were cultured in the presence of *f*-CNT **1**.[23–25] The ammonium functionalized CNT **1** were observed by TEM microscopy and localized inside the cells (Figure 1).[26] Radioactive-labeled carbon nanotubes, derived from *f*-CNT **1**, were intravenously injected in mice and found to be excreted in urine,[19] demonstrating that functionalized, water-soluble CNT can be well-tolerated in vivo, while exhibiting a unique capacity to cross cell membranes and localize into the cytoplasm.[23–25]

Strictly related to this phenomenon is the study of the mechanism leading to cellular uptake of nanotubes. Even though many different laboratories have now independently obtained evidence of carbon nanotube cellular internalization, the exact mechanism responsible for such observations is still a matter of debate.[24,27–29] We believe that different pathways are responsible for cellular uptake of nanotubes strongly dependent on the type of nanotubes and the type of biomolecules on their surface. The CNT functionalized covalently with small molecular weight molecules seem to penetrate plasma membranes to a considerable extent via an energy-independent mechanism and cross the membrane in a passive way acting like tiny needles.[24,26] In particular, we have found that all the nanotubes reported in Table 1 can be uptaken by cells to a considerable degree in an energy-independent manner. The extent of such cell penetrating capacity of *f*-CNT is not yet determined and is currently under further investigation in our laboratories. However, we have already started seeing other laboratories independently obtaining almost identical observations to our original "nanoneedle" penetration capacity of individualized carbon nanotubes using different types of CNT, further reinforcing our proposed hypothesis.[30]

Functionalized Carbon Nanotubes for Gene Delivery

Since *f*-CNT **1** are cationic under physiological conditions, they were also found to efficiently complex[31] and translocate DNA inside cells[26,32] in the first studies exploring the capability of *f*-CNT to deliver genes. We have produced the supramolecular complexes between *f*-CNT **1** and plasmid DNA which express the β-galactosidase marker gene. The expression of the marker gene using *f*-CNT **1** reached 5–10 times higher levels than the plasmid DNA delivered alone, which allows consideration of this novel approach for gene delivery as promising. The potential of gene therapeutics based on carbon nanotubes has been further explored for gene silencing applications.[33,34] Complexes of single-walled carbon nanotubes with siRNA strands modified with a hydrocarbon tail were used to target and specifically kill cancer cells. Further developments of this system are however necessary to validate the in vivo use of this methodology.

TABLE 1. Molecular Structures of the Carbon Nanotube Conjugated with Different Therapeutic Agents

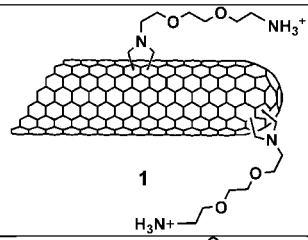
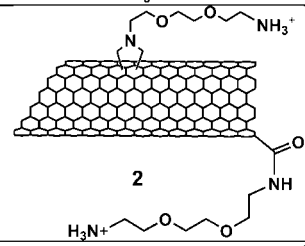
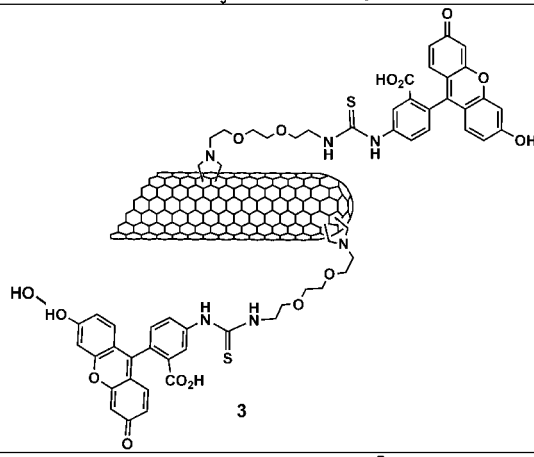
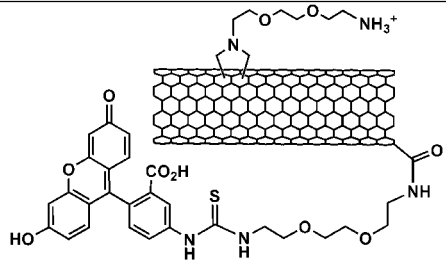
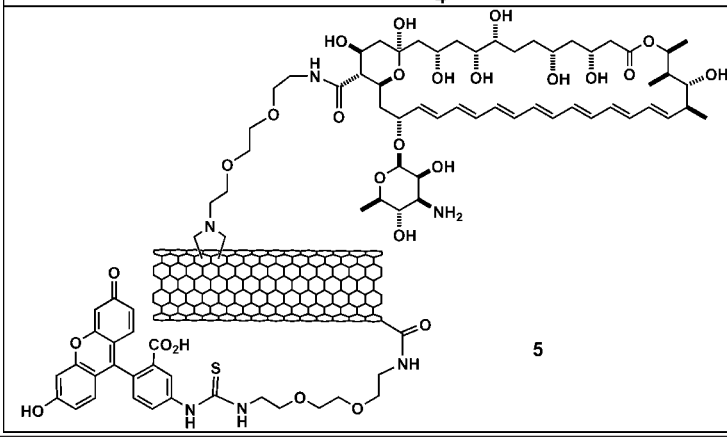
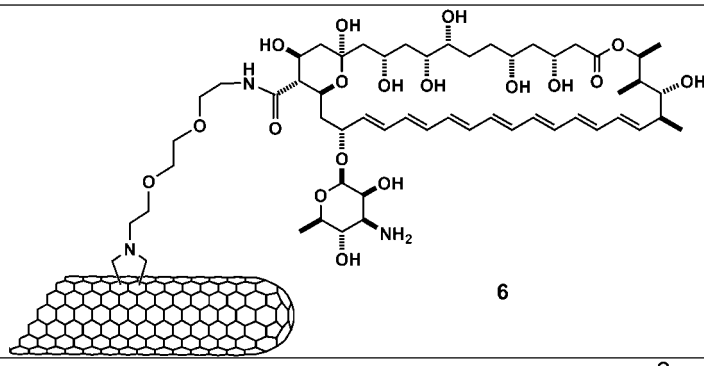
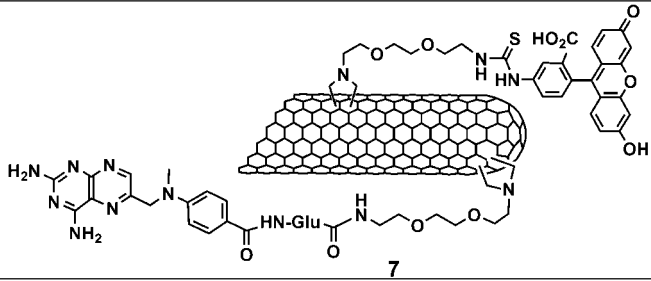
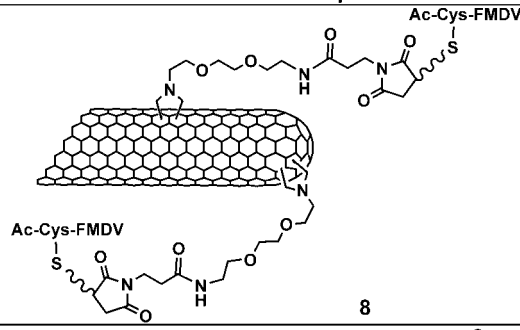
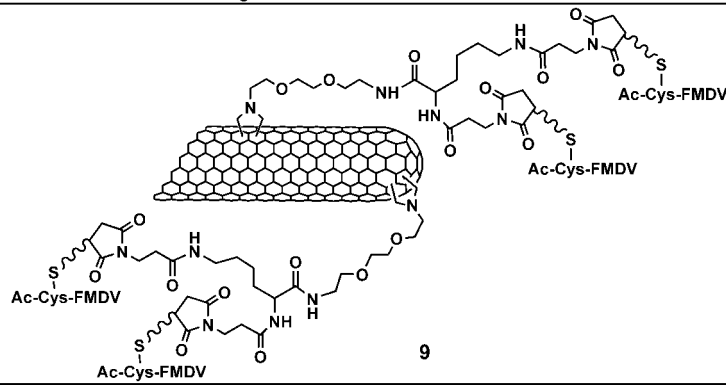
Compounds	Bioassays
 **1**	Cell internalization[24, 26, 27] Intracellular trafficking[24, 27] Cell viability[26] Plasmid DNA delivery[26, 32]
 **2**	Precursor for the preparation of CNT **4** and **5**
 **3**	Cell internalization[23-25] Intracellular trafficking[23-25] Cell viability[23-25]
 **4**	Cell internalization[24]
 **5**	Cell internalization[22, 24] Cell viability[22]

TABLE 1. Continued

Compounds	Bioassays
 6	Antibiotic delivery[22]
 7	Cell internalization[36] Cell viability[36] Anticancer delivery[36]
 8	Immunogenic activity[41, 42] (FMDV peptide corresponds to the 141-159 region of the viral envelope protein VP1 from foot-and-mouth disease virus)
 9	Immunogenic activity[42] (FMDV peptide corresponds to the 141-159 region of the viral envelope protein VP1 from foot-and-mouth disease virus)

Functionalized Carbon Nanotubes for Infectious Diseases

In another approach, carbon nanotubes could be functionalized with antibiotics. Amphotericin B is an antimycotic agent used against particularly resistant fungal strains.[35] It is, however, of limited use because it is highly toxic to mammalian cells, likely due to its low solubility in water and its tendency to aggregate and form pores in the cell membrane. We reasoned that the conjugation of amphotericin B to carbon nanotubes could modulate its properties in terms of toxicity and antimycotic efficiency.[22] The first issue we addressed with *f*-CNT **5** was the cytotoxicity against mammalian cells. We found that, whereas amphotericin B is highly toxic at 10 μg/mL concentration, reaching 40% cell mortality, CNT-conjugated AmB **5**, used at increasing concentrations (up to 40 μg/mL, corresponding to a concentration of amphotericin B

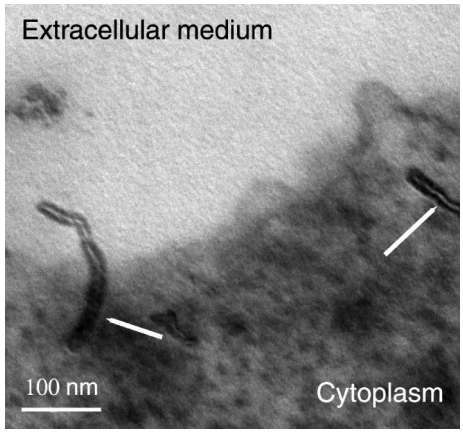


FIGURE 1. Ultrathin transverse section of a HeLa cell treated with ammonium-functionalized CNT **1**. Carbon nanotubes are crossing the cell membrane or visible into the cytoplasm (white arrows).

linked to the tubes of 10 μg/mL), was not toxic. Next, we investigated the cell penetration ability of *f*-CNT **5** bearing both amphotericin B and fluorescein. This latter component allows for the detection of *f*-CNT **5** in the cells. The fluorescence was clearly evident inside the cell compartments. At the same time, the toxicity against fungi and yeasts was enhanced. Preliminary data (kindly provided by Prof. R. Gennaro and Dr. M. Benincasa, University of Trieste) show that *f*-CNT **6** are also active on strains usually resistant to amphotericin B. The reason for this enhanced therapeutic effect is not totally clear. A more detailed study is currently underway to elucidate whether the mechanism of action of *f*-CNT **6** is similar to that of amphotericin B itself or whether it differs in some aspects, then accounting also for the reduced toxicity to mammalian cells.

Functionalized Carbon Nanotubes for Oncology

f-CNT **7** contains a methotrexate molecule together with a fluorescein probe.[36] Methotrexate is a well-known and potent anticancer agent, used also to cure autoimmune diseases.[37] However, methotrexate suffers of low bioavailability and toxic side effects.[38] Therefore, an increased bioavailability and a targeted delivery are highly desirable. *f*-CNT **7** might offer the possibility for improving bioavailability and, in the presence of a targeting unit, to address specifically cancer cells. Preliminary results have shown that methotrexate conjugated to the nanotubes is as active as methotrexate alone in a cell culture assay where Jurkat cells were incubated up to 72 h (unpublished data). The reason for lack of enhanced efficacy between the *f*-CNT **7** and the nonconjugated drug could be attributed to the stable amide bond between methotrexate and the nanotubes. Indeed, the drug is probably released too slowly from the tubes into the cytoplasm for an efficient interaction with its receptor. It is certainly necessary to introduce a cleavable linker or a more enzymatically sensitive bond in the CNT **7** as demonstrated for conjugates based on dendrimers.[39] However, *f*-CNT **7** can be considered a promising construct, and we are currently improving its activity. Following an alternative approach, carbon nanotubes modified with a carborane moiety have been developed for cancer applications.[40] Single-walled CNT were functionalized with a substituted carborane cage for boron neutron capture therapy. The biodistribution study on different tissues showed that water-soluble carborane nanotubes were concentrated more in tumor cells than in other organs when administered intravenously. These results were preliminary although also promising for future applications of carbon nanotube boron-based agents for effective treatment of cancer.

Functionalized Carbon Nanotubes for Vaccination

Another class of carbon nanotube-based therapeutic candidates consists of their constructs with synthetic peptide for immune system activation. The decoration of functionalized nanotubes with B and T cell peptide epitopes can generate a multivalent system able to induce a strong immune response.[41,42] Peptides can be connected to the tubes using the chemoselective approach.[43] This strategy is based on the preparation of functionalized carbon nanotubes with a maleimide group which readily reacts with peptides containing a cysteine residue at one end. The thiol group of the cysteine selectively adds to the maleimide forming a stable covalent bond. The advantage of this method is that the peptide, obtained by solid phase synthesis, is fully deprotected and characterized before performing the ligation to the nanotubes. *f*-CNT **8** and **9** were prepared following this type of chemistry by linking a B cell epitope from a coat protein of foot-and-mouth disease virus (FMDV). The two conjugates differ in the amount of peptide around the nanotubes which was doubled for *f*-CNT **9** using a lysine branch. The antigenic and immunogenic properties of these conjugates were measured. In particular, *f*-CNT **8** and **9** elicited high antibody responses in comparison to the nonconjugated peptide. In addition, the generated antibodies had the capacity to neutralize the virus, thus demonstrating the potential of carbon nanotubes as components for synthetic vaccine development.

Conclusions

Besides the advances in novel synthetic chemistry that have led to the design and discovery of multiple small drug mole-

cules, the product pipelines of pharmaceutical companies suffer from the lack of clinically viable product candidates. The incorporation of nanotechnology tools at early stages in the drug development process may offer new ways of "rediscovering" old drug molecules and also offer new discovery and design options for new molecules. Our laboratories have developed functionalized CNT with the objective of creating a completely new component for drug development. We described different strategies by which carbon nanotubes can be conjugated with many different small molecules or macromolecules that can act as therapeutic agents in order to achieve improved therapeutic efficacy. The current available examples in our laboratories include small molecule anticancer and antibiotic carbon nanotube conjugates as well as antigen-presenting conjugates for the potential fabrication of vaccines and plasmid DNA complexes for construction of gene therapeutics. Although it may be premature at this stage to claim that carbon nanotubes will be clinically successful therapeutics, the results highlighted in this Account can certainly be considered promising. We anticipate carbon nanotubes to play a critical role as exemplary nanomaterials that can be clinically developed and constitute archetypal cases in the emerging field of nanomedicine.

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BIOGRAPHICAL INFORMATION

Maurizio Prato obtained his Laurea degree in chemistry in 1978 from the University of Padova, Italy, where he was appointed Assistant Professor in 1983. He moved to Trieste as an Associate Professor in 1992. He was therefore promoted to Full Professor in 2000. He spent a postdoctoral year in 1986−1987 at Yale University, was Visiting Scientist at the University of California, Santa Barbara, in 1991−1992, and was Professeur Invité at the Ecole Normale Supérieure in Paris, France, in July 2002. His research interests focus on the functionalization chemistry of fullerenes and carbon nanotubes for applications in materials science and medicinal chemistry and on the synthesis of biologically active substances. His scientific contributions have been recognized by national awards, which include Federchimica Prize (1995, Association of Italian Industries) and the National Prize for Research (2002, Italian Chemical Society).

Kostas Kostarelos is Chair of Nanomedicine at The School of Pharmacy, University of London and a Fellow of the Royal Society of Medicine (FRSM) and the Institute of Nanotechnology (FIoN). He read chemistry at the University of Leeds, UK, and obtained his Diploma and PhD in Chemical Engineering from Imperial College London. His previous academic appointments include Deputy Director, Imperial College Genetic Therapies Centre, Imperial College London, UK; Assistant Professor of Genetic Medicine & Chemical Engineering in Medicine, Cornell University Weill Medical College, NY, USA; Manager, Bioengineering Core, Belfer Gene Therapy Center, Cornell University Weill Medical College, NY, USA; Instructor, Pulmonary & Critical Care Medicine, New York-Presbyterian Hospital, NY, USA. He is the Senior Editor of the journal *Nanomedicine* and a Senior Founding Member of the *American Academy of Nanomedicine* (Washington, DC, USA).

Alberto Bianco received his Laurea degree in Chemistry in 1992 and his PhD in 1995 from the University of Padova (Italy), under the supervision of Professor Claudio Toniolo, working on fullerene-based amino acids and peptides. As a visiting scientist, he worked at the University of Lausanne during 1992 (with Professor Manfred Mutter), at the University of Tübingen in 1996−1997 (with Professor Günther Jung, as an Alexander von Humboldt fellow), and at the University of Padova in 1997−1998 (with Professor Gianfranco Scorrano). He currently is a Research Director at CNRS in Strasbourg (France). His research interests focus on the development of carbon-based nanomaterials (carbon nanotubes and fullerenes) and their use as therapeutic vectors; the applications of functionalized carbon nanotubes and fullerenes in nanomedicine; the synthesis of peptidomimetics containing fullero-amino acids as new ligands for immunotherapy; and the organic and combinatorial solid phase synthesis and characterization of the molecules on the solid support by HRMAS NMR. He is member of the American Chemical Society, the French Group of Peptides and Proteins, and the European Peptide Society.

FOOTNOTES

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