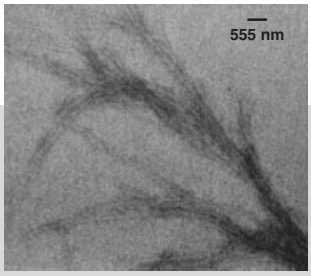
Can Carbon Nanotubes Be Considered Useful Tools for Biological Applications?**

By *Alberto Bianco** and *Maurizio Prato**



Carbon nanotubes can be made soluble in both organic solvents and in aqueous solutions by organic functionalization. In particular, soluble carbon nanotubes can be further derivatized by coupling with amino acids and bioactive peptides. Immobilization of peptides to the external walls of carbon nanotubes may find interesting applications in diagnostics, vaccine and drug delivery, or multipresentation of bioactive molecules.

1. Introduction

The exceptional interest in carbon nanotubes (CNTs) resides in their possible technological applications in various fields of science. For instance, CNTs are considered the strongest materials known and hold strong promise as nanoelectronic components.[1] However, although new ways of handling CNTs are progressively becoming available,[2] the ability to solubilize and separate individual CNTs is still a great challenge.[3] A very general way to achieve this is by organic functionalization. In fact, the covalent attachment of organic moieties to CNTs may lead to very interesting objects with new applications in materials science and medicinal chemistry.[4–6] Until now there have been only very few reports describing biological applications of CNTs.[5,6] This is mainly due to the complete lack of solubility of CNTs under physiological conditions.

The field of functionalization of CNTs is currently expanding. Several different ways have been considered and are under active investigation. Exhaustive as well as critical reviews have appeared in the literature recently.[1b,7] Herein, we focus on covalent attachment of bioactive molecules to CNTs, mainly as achieved in our laboratories. Our main scope is to demonstrate that CNTs can serve as useful scaffolds for new nanobiotechnological applications.[8]

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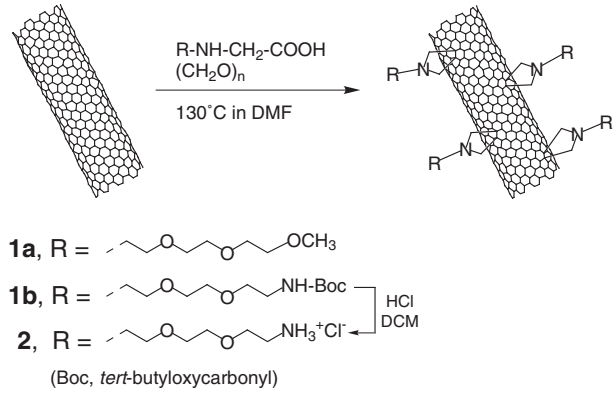
2. Synthesis

We have recently reported that CNTs undergo 1,3-dipolar cycloaddition when heated in DMF in the presence of an α-amino acid and an aldehyde.[9] The scope of the reaction is very broad and provides functionalized CNTs (f-CNTs) that exhibit extraordinary solubility in organic solvents and even in aqueous solutions (e.g., f-CNT **1a** has a solubility in chloroform of 50 mg mL^{-1}, Scheme 1). The enhanced solubility allows several physico-chemical studies previously impossible



Scheme 1. Dipolar cycloaddition to CNTs of azomethine ylides generated by condensation of an α-amino acid and *p*-formaldehyde.

for CNTs. For instance, ^{1}H-NMR of f-CNTs can be easily recorded, which shows relatively broad bands for protons close to the CNTs, becoming sharper for more distant protons. This is expected on the basis of the different magnetic environments that the protons experience when close to the curved graphitic region. On the other hand, when far from the CNT electron cloud the organic moiety is free to move and rotate, making the signal more resolved.

Virtually any functional group can be attached to CNTs, so that f-CNTs are now available for technological applications. For example, f-CNTs possessing several thousands of active

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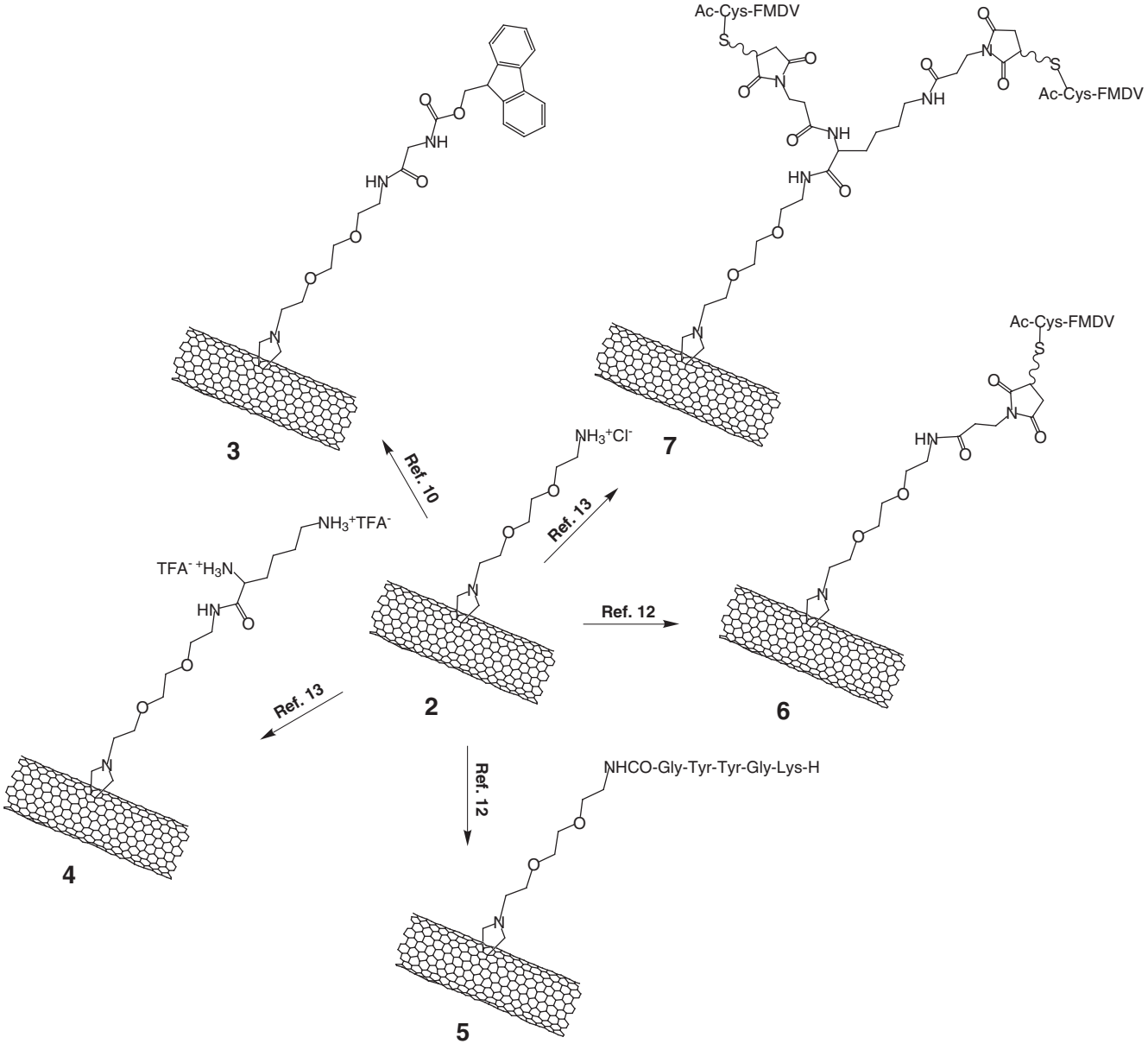
groups can be further functionalized with biologically active moieties. To extend the use of CNTs in biology and medicinal chemistry, it is necessary to prepare a material soluble in physiological solutions. For this purpose, f-CNT **2** (Scheme 2) modified with an ammonium-terminated triethylene glycol chain was prepared from the corresponding Boc-protected amine (**1b**, Fig. 1). f-CNT **2** is highly soluble in aqueous solutions (>20 mg mL^{-1}).[10] No precipitation due to aggregation phenomena was observed after storing f-CNT **2** solution for over one month. The amount of amino groups homogeneously distributed at the ends and around the side walls of the wires was determined using the quantitative Kaiser test, a common practice in peptide chemistry.[11] A loading (number of millimoles of amino groups per gram of material) in the range 0.3–0.5 mmol g^{-1} was achieved, typical of the resins for solid-phase peptide synthesis.[11] f-CNT **2** was tested to prove its utility in peptide synthesis and was therefore functionalized with N-terminal protected amino acids. f-CNT **3** bearing a ^{15}N-protected glycine residue was obtained, which was fully characterized by spectroscopic and microscopic techniques (Scheme 2).[10] Soon after, we were able to attach a series of amino acids and bioactive peptides to the amino functions of the tubes, to obtain conjugates **4**–**7** (Scheme 2). The C-terminus of a fully-protected pentapeptide was coupled, via the fragment condensation strategy, to the amino groups of f-CNT **2**, affording peptide–CNT conjugate **5** (Fig. 1).[12] The formation of a covalent bond between the CNT and the peptide was confirmed by two-dimensional (2D) NMR experiments, so that all the spin systems of the peptide moiety were identified.



Scheme 2. Amino acid and peptide functionalization of CNTs.

ADVANCED MATERIALS

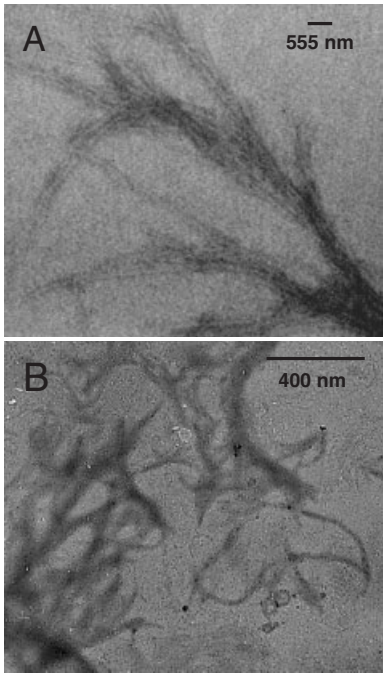


Fig. 1. Transmission electron microscopy image of the tangled bundles of A) f-CNT **2** and B) peptide–CNT **5**.

f-CNT **2** was also modified by coupling a maleimido linker. This group allows a selective chemical ligation of peptides, provided that a cysteine residue with a free thiol is present. The conjugation of peptides using this strategy was demonstrated using the epitope of 19 amino acids from the VP1 envelop protein of the foot-and-mouth disease virus (FMDV). A necessary cysteine residue was inserted at the N-terminus to allow chemical ligation. Peptide–CNT **6** was obtained, which represents the first example of a bioactive peptide covalently linked to CNTs.[12] More recently, we have prepared CNT **4** containing an N-terminal and side chain N-deprotected lysine that increases the water solubility of the tubes (Scheme 2). The bifunctional amino acid residue permits the introduction of two copies of the peptide as shown for conjugate **7** (Scheme 2).[13] Again, we used the selective chemical ligation through a maleimido linker to bind FMDV peptide to the nanotube. f-CNTs **6** and **7** were tested for their biological activity, and in particular their immunological properties (see below).

3. Biological Properties

Up until now only pristine CNTs have been used in the field of peptide chemistry: 1) to selectively bind amphiphilic helical peptides through van der Waals interactions[14] and 2) to discriminate between hydrophobic peptides derived from phage displayed peptide libraries.[15] We have reasoned that the covalent binding of bioactive peptides to CNTs would have interesting applications in vaccine delivery. Initially, our goal was to study the ability of such systems to stimulate antigen-specific immune responses. Indeed, we were able to demonstrate that peptide–CNTs are recognized by specific monoclonal and polyclonal antibodies and induce high titers of antibodies following different immunization protocols. For this study, we chose a B-cell epitope corresponding to the sequence ^{141}Gly-Ser-Gly-Val-Arg-Gly-Asp-Phe-Gly-Ser-Leu-Ala-Pro-Arg-Val-Ala-Arg-Gln-Leu^{159} from VP1 protein of FMDV.[16] This peptide was a very promising candidate for vaccine formulation because it represents a virus neutralizing and protective epitope.[17] The antigenicity, namely the capacity of an antibody to recognize the peptide attached onto the CNT, and the immunogenicity, corresponding to the capacity of peptide–CNTs to elicit peptide-specific antibodies, were studied. The antigenic characteristics of both peptide–CNT conjugates **6** and **7** have been evaluated using surface plasmon resonance (SPR, BIAcore technology) and enzyme-linked immunosorbent assays (ELISA). The SPR technique allows the study of antigen-antibody interactions in real time. A specific anti-FMDV 141-159 peptide monoclonal antibody (mAb) was fixed to a gold chip via a second antibody. A solution of free FMDV peptide, acetylated CNTs **2**, or CNT-linked peptides **6** and **7** was passed over the immobilized mAb to measure the increase in mass due to the interaction between the different entities (Fig. 2). CNTs without any bound peptide did not react with the mAb, whereas free peptide, mono-conjugate **6**, and bis-conjugate **7** interacted with increasing mass. The results obtained from these measurements suggested that the peptide linked to the CNT retains its structural characteristics for recognition by the specific antibody.[12,13] This was further confirmed by coating the functionalized CNTs on ELISA plates and allowing them to react with the specific antibodies. This experiment confirmed that the secondary structure of the nanotube-linked peptide, necessary for the spatial interaction with the antibody, is properly presented by the carbon wires.[12] The immunogenicity of peptide–CNTs conjugates **6** and **7** was subsequently evaluated by a series of experiments in vivo.[13] Immunization of mice with **6** and **7** enhanced anti-

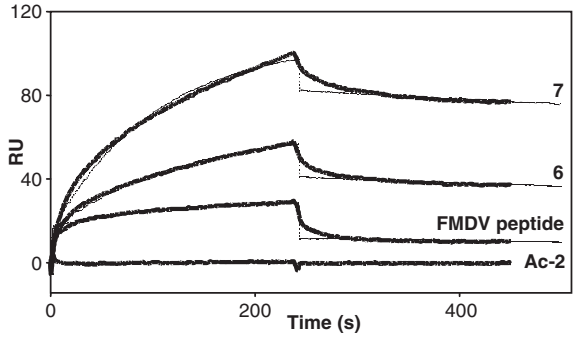


Fig. 2. Sensorgrams obtained by allowing the analytes to react with an anti-peptide mAb. The association phase required 4 min, the dissociation phase 5 min. Response with peptide–CNT **7**, peptide–CNT **6**, free FMDV peptide, and acetylated CNT **2** (Ac-**2**) at 5 μM concentration (bold lines). Thin lines show the theoretical curves (Langmuir 1:1) for peptide–CNT **7** (K_d = 170 nM), peptide–CNT **6** (K_d = 300 nM), and free FMDV peptide (K_d = 350 nM). RU value corresponds to the resonance unit (1000 RU = 1 ng mm^{-2} of analyte).

FMDV peptide antibody responses as compared to the free peptide. The antibodies were peptide-specific and were not directed to the peptide CNT linker. Most importantly, no antibodies against f-CNT **2** devoid of peptide moiety were detectable. The lack of immune response to CNT is of fundamental importance in view of their use as a carrier system for antigens. Normally, the low immunogenicity of peptide antigens is improved by coupling them to carrier proteins. However, such systems could influence the immune response to the linked peptide by causing the phenomenon of epitopic suppression (e.g., high titers of non-specific antibodies are generated against the carrier protein).[18]

4. Conclusion and Perspectives

Functionalized CNTs hold a lot of promise for applications in the field of medicinal chemistry. In particular, conjugation of bioactive peptides to the external walls of the tubes allows to prepare bioactive materials endowed of immunological properties. The development of new systems based on CNT scaffolds to target different issues can be envisaged. Besides delivery of candidate vaccine antigens, other applications, including delivery of drugs, peptidomimetics, proteins, oligonucleotides, can be achieved using CNTs. Conjugation of plasmid DNA would open the way to gene therapy. In addition, CNT-displayed peptides have several advantages for use in diagnostics: i) their efficient binding on ELISA plates overcomes potential problems encountered with direct coating of peptides onto a solid support due to their physico-chemical properties; ii) they present peptides in a more accessible way for antibody recognition in comparison to peptides coated directly onto the ELISA plate; iii) they have the potential to present different epitopes thus allowing higher diagnostic accuracy. The possibility of creating multifunctionalized CNTs for the selective binding of different peptides may also represent an area of further development.

[1] a) M. S. Dresselhaus, G. Dresselhaus, P. Avouris, *Carbon Nanotubes: Synthesis, Properties and Applications*, Springer-Verlag, Berlin **2001**. b) Special issue on Carbon Nanotubes, *Acc. Chem. Res.* **2002**, *35*, 997.

[2] T. Fukushima, A. Kosaka, Y. Ishimura, T. Yamamoto, T. Takigawa, N. Ishii, T. Aida, *Science* **2003**, *300*, 2072.

[3] a) C. Richard, F. Balavoine, P. Schultz, T. W. Ebbesen, C. Mioskowski, *Science* **2003**, *300*, 775. b) A. Star, D. Steuerman, J. R. Heath, J. F. Stoddard, *Angew. Chem. Int. Ed.* **2002**, *41*, 2508. c) W. Zhao, C. Song, P. E. Pehrsson, *J. Am. Chem. Soc.* **2002**, *124*, 12 418.

[4] a) P. M. Ajayan, *Chem. Rev.* **1999**, *99*, 1787. b) A. B. Dalton, S. Collins, E. Muñoz, J. M. Razal, V. Howard Ebron, J. P. Ferraris, J. N. Coleman, B. G. Kim, R. H. Baughman, *Nature* **2003**, *423*, 703. c) Y. Kang, T. A. Taton, *J. Am. Chem. Soc.* **2003**, *125*, 5650. d) K. Jiang, Q. Li, S. Fan, *Nature* **2002**, *419*, 801. e) A. A. Mamedov, N. A. Kotov, M. Prato, D. M. Guldi, J. P. Wicksted, A. Hirsch, *Nat. Mater.* **2002**, *1*, 190. f) B. R. Azamian, J. J. Davis, K. S. Coleman, C. B. Bagshaw, M. L. Green, *J. Am. Chem. Soc.* **2002**, *124*, 12 664.

[5] a) S. E. Baker, W.Cai, T. L. Lasseter, K. P. Weidkamp, R. J. Hamers, *Nano Lett.* **2002**, *2*, 1413. b) K. A. Williams, P. T. M. Veenhuizen, B. G. de la Torre, R. Eritjia, C. Dekker, *Nature* **2002**, *420*, 761. c) H. Xin, A. T. Woolley, *J. Am. Chem. Soc.* **2003**, *125*, 8710.

[6] a) J. J. Gooding, R. Wibowo, J. Liu, W. Yang, D. Losic, S. Orbons, F. J. Mearns, J. G. Shapter, D. B. Hibbert, *J. Am. Chem. Soc.* **2003**, *125*, 9006. b) R. J. Chen, Y. Zhang, D. Wang, H. Dai, *J. Am. Chem. Soc.* **2001**, *123*, 3838. c) R. J. Chen, S. Bangsaruntip, K. A. Drouvalakis, N. Wong Shi Kam, M. Shim, Y. Li, W. Kim, P. J. Utz, H. Dai, *Proc. Natl. Acad. Sci. USA* **2003**, *100*, 4984.

[7] a) A. Hirsch, *Angew. Chem. Int. Ed.* **2002**, *41*, 1853. b) T. Lin, V. Bajpai, T. Ji, L. Dai, *Aust. J. Chem.* **2003**, *56*, 635. c) J. L. Bahr, J. M. Tour, *J. Mater. Chem.* **2002**, *12*, 1952. d) S. Banerjee, M. G. C. Kahn, S. S. Wong, *Chem. Eur. J.* **2003**, *9*, 1898. e) D. Tasis, N. Tagmatarchis, V. Georgakilas, M. Prato, *Chem. Eur. J.* **2003**, *9*, 4000.

[8] a) M. C. Roco, *Curr. Opin. Biotechnol.* **2003**, *14*, 337. b) D. F. Emerich, C. G. Thanos, *Expert Opin. Biol. Ther.* **2003**, *3*, 655.

[9] V. Georgakilas, K. Kordatos, M. Prato, D. M. Guldi, M. Holzinger, A. Hirsch, *J. Am. Chem. Soc.* **2002**, *124*, 760.

[10] V. Georgakilas, N. Tagmatarchis, D. Pantarotto, A. Bianco, J.-P. Briand, M. Prato, *Chem. Commun.* **2002**, 3050.

[11] M. Goodman, A. Felix, L. Moroder, C. Toniolo, *Methods of Organic Chemistry (Houben-Weyl)*, Vol. E22a, Thieme, Stuttgart **2002**.

[12] D. Pantarotto, J. Hoebeke, R. Graff, C. D. Partidos, J.-P. Briand, M. Prato, A. Bianco, *J. Am. Chem. Soc.* **2003**, *125*, 6160.

[13] D. Pantarotto, C. D. Partidos, J. Hoebeke, F. Brown, E. Kramer, J.-P. Briand, S. Muller, M. Prato, A. Bianco, *Chem. Biol.* **2003**, *10*, 961.

[14] G. R. Dieckmann, A. B. Dalton, P. A. Johnson, J. Razal, J. Chen, G. M. Giordano, E. Muñoz, I. H. Musselman, R. H. Baughman, R. K. Draper, *J. Am. Chem. Soc.* **2003**, *125*, 1770.

[15] S. Wang, E. S. Humphreys, S.-Y. Chung, D. F. Delduco, S. R. Lustig, H. Wang, K. N. Parker, N. W. Rizzo, S. Subramoney, Y.-M. Chiang, A. Jagota, *Nat. Mater.* **2003**, *2*, 196.

[16] a) D. J. Rowlands, B. E. Clarke, A. R. Carroll, F. Brown, B. H. Nicholson, J. L. Bittle, R. A. Houghten, R. A. Lerner, *Nature* **1983**, *306*, 694. b) L. L. France, P. G. Piatti, J. F. Newman, I. Toth, W. A. Gibbons, F. Brown, *Proc. Natl. Acad. Sci. USA* **1994**, *91*, 8442.

[17] M. J. Francis, *Sci. Prog.* **1990**, *74*, 115.

[18] M. P. Schutze, C. Leclerc, M. Jolivet, F. Audibert, L. Chedid, *J. Immunol.* **1985**, *135*, 2319.

