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Applications of carbon nanotubes in drug delivery

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The development of new and efficient drug delivery systems is of fundamental importance to improve the pharmacological profiles of many classes of therapeutic molecules. Many different types of drug delivery systems are currently available. Within the family of nanomaterials, carbon nanotubes (CNT) have emerged as a new alternative and efficient tool for transporting and translocating therapeutic molecules. CNT can be functionalised with bioactive peptides, proteins, nucleic acids and drugs, and used to deliver their cargos to cells and organs. Because functionalised CNT display low toxicity and are not immunogenic, such systems hold great potential in the field of nanobiotechnology and nanomedicine.

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Introduction

Carbon nanotubes (CNT) [1–3] are considered ideal materials for several applications [4], ranging from ultra-strong fibers [5] to field emission displays [6]. Recently, CNT have generated great interest in biology [7•], where suitably modified CNT can serve as vaccine delivery systems [8] or protein transporters [9•].

CNT can be imaginatively produced by rolling up a single layer of graphene sheet (single-walled CNT; SWNT) [10,11], or by rolling up many layers to form concentric cylinders (multi-walled CNT; MWNT) [3] (Figure 1). As-produced CNT, both SW and MW, are commercially available, with different structural details and variable degrees of purity. Pristine CNT are completely insoluble in all solvents, which has generated some health concerns; consequently, their biological properties are being studied in terms of toxicity [12]. The development of efficient methodologies for the chemical modification of CNT has stimulated the preparation of soluble CNT that can be employed in several biological applications, among which drug delivery appears to be particularly promising [13•,14–16].

Two functionalisation approaches are widely employed for modification of CNT (Figure 2). CNT can be oxidised using strong acids, resulting in the reduction of their length while generating carboxylic groups, which increase their dispersibility in aqueous solutions [17]. Alternatively, addition reactions to the CNT external walls and tips make them soluble in water [18,19]. Solubility under physiological conditions is a key prerequisite to make CNT biocompatible. In addition, functionalised carbon nanotubes (*f*-CNT) can be linked to a wide variety of active molecules, including peptides, proteins, nucleic acids and other therapeutic agents.

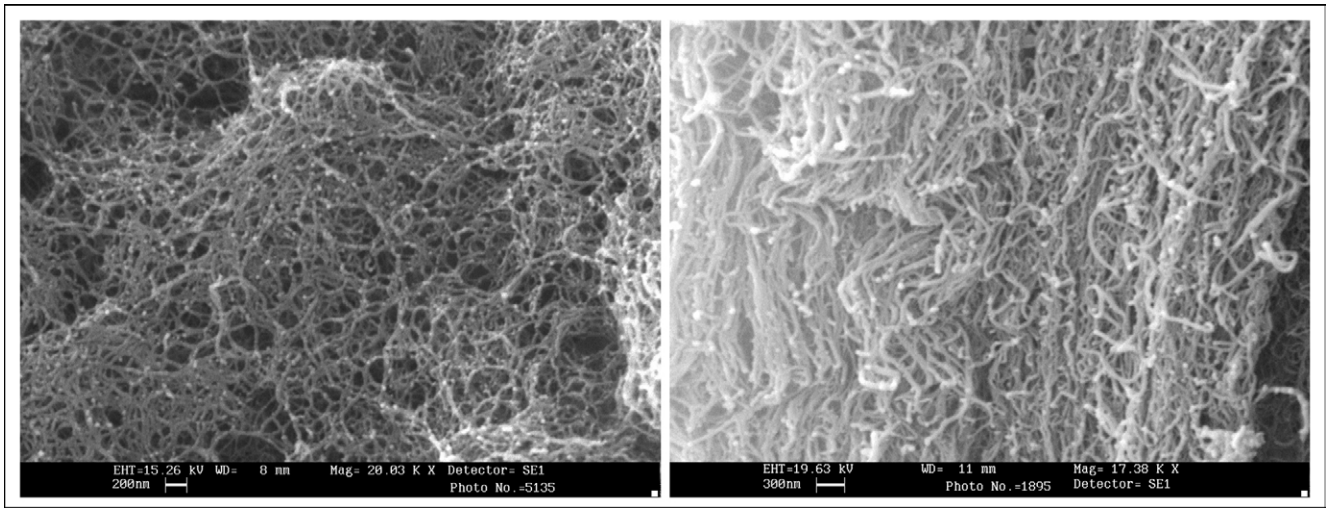
An efficient way to functionalise the external walls of CNT is based on the 1,3-dipolar cycloaddition of azomethine ylides. CNT undergo the addition reaction when heated in DMF in the presence of an α-amino acid and an aldehyde [20]. The scope of this reaction is very broad and produces *f*-CNT that possess high solubility in a wide range of solvents. By carefully choosing the reactants, it is possible to modulate solubility in organic solvents or aqueous solutions [21•]. CNT carrying ammonium groups (Figure 2b) are very soluble in water and have been exploited for their potential in the delivery of therapeutic molecules.

The biological applications of *f*-CNT are currently under intense investigation. In this paper we review the most recent achievements of CNT in drug delivery, with a specific emphasis on the work performed in our groups.

Peptide delivery by carbon nanotubes

We initially studied the application of CNT as a template for presenting bioactive peptides to the immune system [22]. For this purpose, a B-cell epitope of the foot-and-mouth disease virus (FMDV) was covalently attached to the amine groups present on CNT, using a bifunctional linker. The peptides around the CNT adopt the appropriate secondary structure for recognition by specific monoclonal and polyclonal antibodies. The immunogenic features of peptide–CNT conjugates were subsequently assessed *in vivo* [23]. Immunisation of mice with FMDV peptide–nanotube conjugates elicited high antibody responses as compared with the free peptide. These antibodies were peptide-specific since antibodies against CNT were not detected. In addition, the antibodies

Figure 1



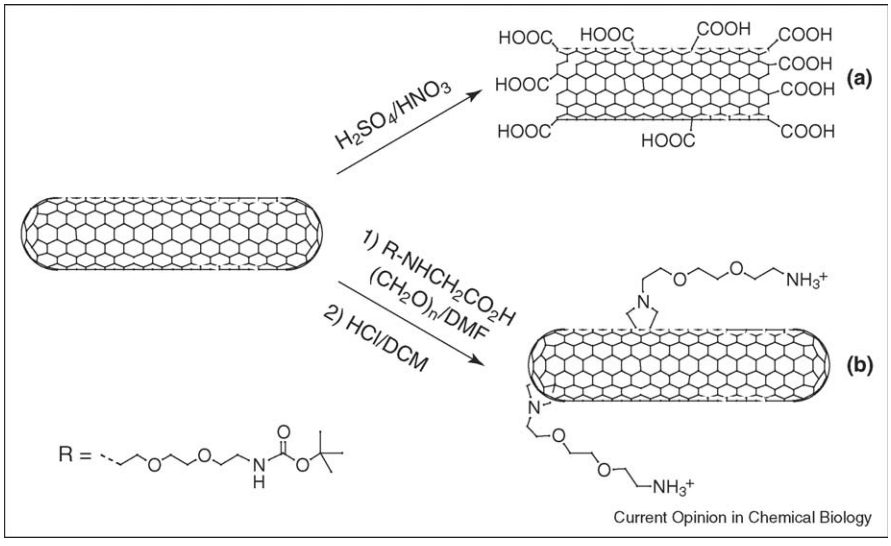
Scanning electron microscopy (SEM) images of pristine single- (left) and multi-walled (right) carbon nanotubes.

displayed virus-neutralising ability. The use of CNT as potential novel vaccine delivery tools was validated by interaction with the complement [24]. The complement is that part of the human immune system composed of a series of proteins responsible for recognising, opsonising, clearing and killing pathogens, apoptotic or necrotic cells and foreign materials. Salvador-Morales *et al.* [24] showed that pristine CNT activate the complement following both the classical and the alternative way by selective adsorption of some of its proteins. Because complement activation is also involved in immune response to antigens, this might support the enhancement of antibody response following immunisation with peptide–CNT conjugates.

Cellular uptake of carbon nanotubes

An important characteristic of *f*-CNT is their high propensity to cross cell membranes [25•,26•]. CNT labelled with a fluorescent agent were easily internalised and could be tracked into the cytoplasm or the nucleus of fibroblasts using epifluorescence and confocal microscopy [25•]. The mechanism of uptake of this type of *f*-CNT

Figure 2

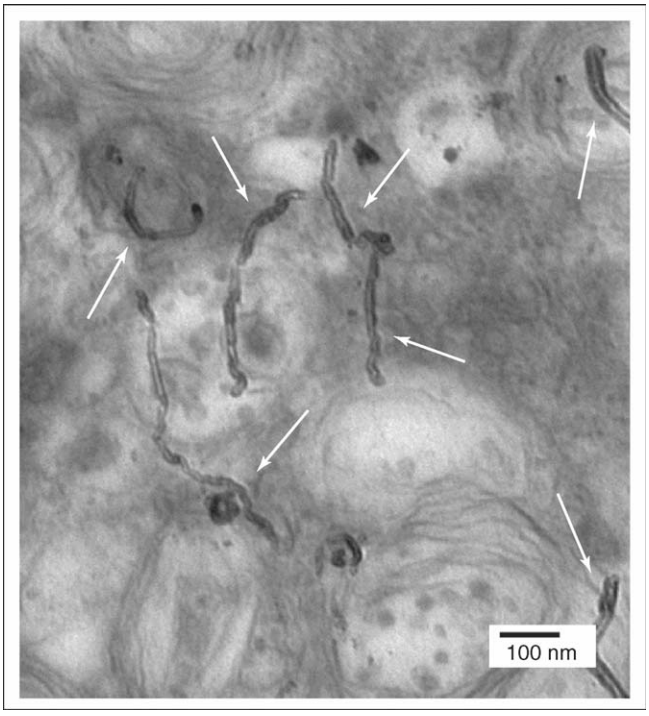


Organic functionalisation of carbon nanotubes. Pristine single- or multi-walled carbon nanotubes can be **(a)** treated with acids to purify them and generate carboxylic groups at the terminal parts, or **(b)** reacted with amino acid derivatives and aldehydes to add solubilising moieties around the external surface.

appears to be passive and endocytosis-independent. Incubation with cells in the presence of endocytosis inhibitors did not influence the cell penetration ability of *f*-CNT. Furthermore, *f*-CNT showed similar behaviour when incubation with the cells was carried out at lower temperatures. Cellular uptake was confirmed by Dai and colleagues [9•,26•] who in later studies used oxidised CNT to covalently link fluorescein or biotin, allowing for a biotin–avidin complex formation with fluorescent streptavidin. Again the nanotubes were observed inside the cells. In this case, the protein–CNT conjugates were found in endosomes, suggesting an uptake pathway via endocytosis. The CNT can also be visualised inside the cells using transmission electron microscopy (TEM) [27]. Functionalised water-soluble CNT were incubated with HeLa cells. The cells were subsequently embedded into an epoxy resin that was sliced using a diamond microtome. Each slice was mounted on a TEM grid and observed under the microscope. Figure 3 shows a typical example of functionalised MWNT distributed into the cytoplasm.

Some tubes were also identified at the cell membrane during the process of translocation. The conformation of CNT perpendicular to the plasma membrane during uptake suggested a mechanism similar to nanoneedles, which perforate and diffuse through the lipid bilayer of plasma membrane without inducing cell death. Dynamic simulation studies have shown that amphiphilic nanotubes can theoretically migrate through artificial lipid bilayers via a similar mechanism [28]. Nanopenetration was also recently suggested by Cai *et al.*, who proposed an efficient *in vitro* delivery technique called nanotube spearing [29]. MCF-7 breast cancer cells were grown on a substrate and incubated with magnetic CNT. A rotating magnetic field first drove the nanotubes to spear the cells. In a subsequent step, a static field pulled the tubes into the cells. On the basis of SEM images, it seems that the tubes cross the cell membrane like tiny needles. Another efficient way to observe CNT intracellularly was developed by Weismann *et al.*, who used near-infrared fluorescence [30]. They showed that macrophage cells could ingest significant amounts of nanotubes without apparent toxic effects. The internalised tubes remained fluorescent and could be identified at wavelengths beyond 1100 nm. Therefore, there is mounting evidence that *f*-CNT are capable of efficient cellular uptake by a mechanism that has not yet been clearly identified. However, the nature of the functional group at the CNT surface seems to play a determinant role in the mechanism of interaction with cells.

Figure 3



Ultrathin transverse section of HeLa cells treated with functionalised MWNT. After incubation, the cells were fixed, stained, dehydrated and embedded into an epoxy resin (Epon® 812). Ultrathin layers (90 nm thickness) were sliced with a diamond ultramicrotome and inspected under TEM. White arrows indicate the *f*-CNT distributed into the cytoplasm.

Nucleic acid delivery by carbon nanotubes

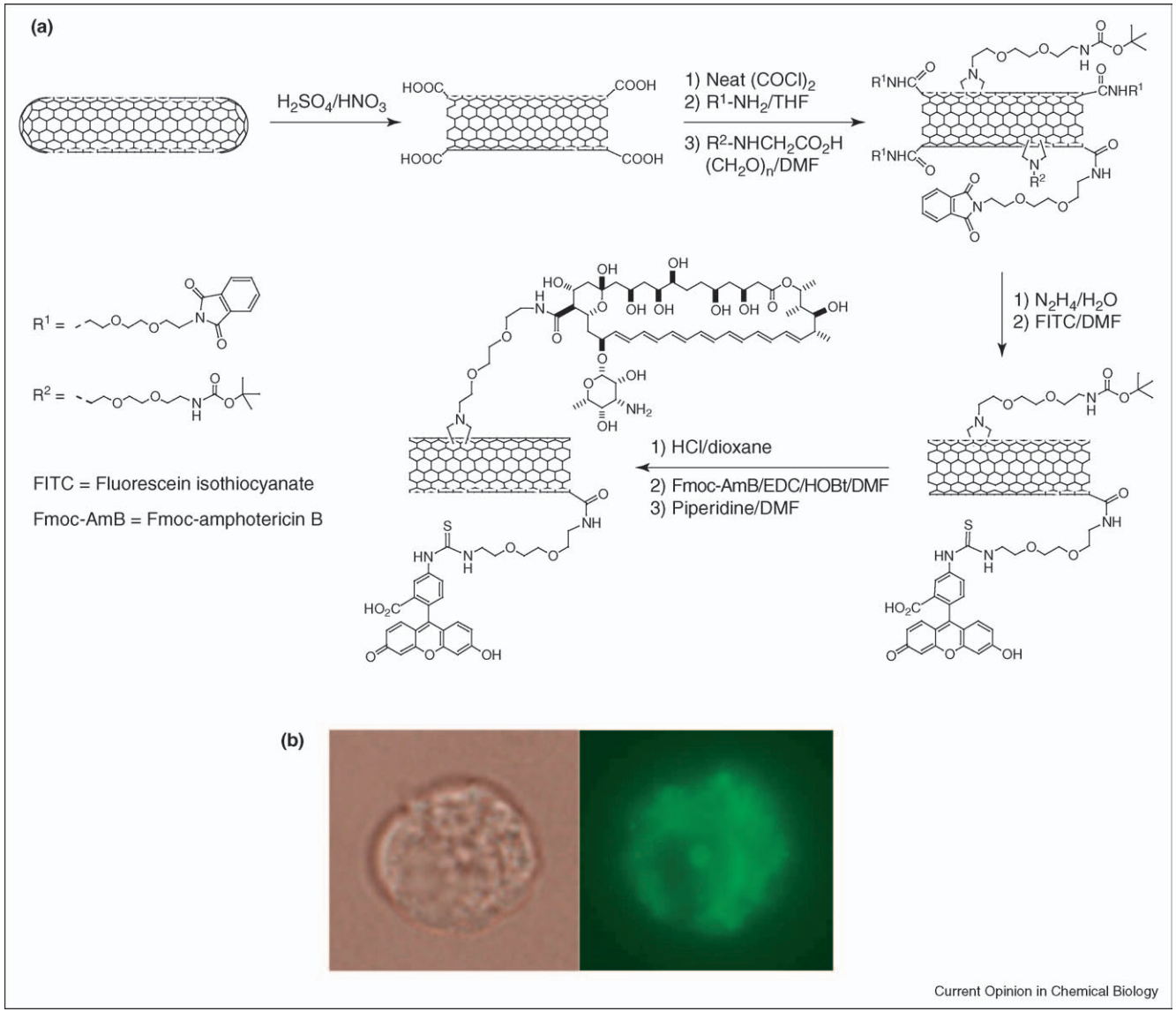
Ammonium-functionalised CNT were tested for their ability to form supramolecular complexes with nucleic acids via electrostatic interactions. Many cationic systems are being investigated for the delivery of nucleic acids to cells [31–33]. Their common goal is to enhance gene transfer and expression, because plasmid DNA alone penetrates into cells and reaches their nucleus with considerable difficulty [34]. Similar to other families of non-viral vectors (i.e. liposomes, cationic polymers, microparticles and nanoparticles), the macromolecular cationic nature of the *f*-CNT has been exploited to condense plasmid DNA [27,35]. To explore the potential of CNT as gene transfer vectors, plasmid DNA pCMV-Bgal expressing β-galactosidase was adsorbed on *f*-CNT carrying ammonium groups. Both single- and multi-walled cationic CNT are able to form stable complexes, characterised by electron microscopy (TEM and SEM), surface plasmon resonance, electrophoresis and fluorescence dye exclusion [35]. Following formation of the complexes, gene transfer experiments showed a clear effect of *f*-CNT on the expression of β-galactosidase [27,35]. Levels of gene expression five to ten times higher than that of DNA alone were obtained. More recently, the efficiency of DNA transfer using *f*-CNT was increased by covalent modification of the external walls of the tubes with polyethyleneimine (PEI) [36]. PEI-grafted MWNT complexed and delivered plasmid DNA to different cell types; however, the measured levels of luciferase expression were similar to that of PEI alone.

Using a similar approach, we demonstrated that cationic carbon nanotubes are able to condense short oligodeoxynucleotide (ODN) sequences and improve their immunostimulating activity [37]. ODNs containing GpG motifs hold much interest for immunotherapy and vaccination. We showed that CpG interacts with ammonium-CNT with no toxicity on mouse splenocytes. More importantly, high ratios of *f*-CNT over a minimum immunostimulatory dose of a specific ODN CpG increased the immunopotentiating activity *in vitro* while decreasing the secretion of proinflammatory cytokine interleukine-6.

CNT were also used to deliver non-encoding RNA polymers into cells [38]. SWNT condensed RNA by non specific binding. Translocation of the complexes between CNT and poly(rU) RNA polymer into MCF-7 cells was assessed by radioisotope labelling and confocal fluorescence. The hybrids showed negligible toxicity as found by monitoring cell growth.

It is evident that CNT can form stable supramolecular assemblies with nucleic acids, thus opening the way to diverse applications including gene therapy, genetic vaccination and immunopotentiation enhancement.

Figure 4



Functionalisation and imaging of CNT with amphotericin B. **(a)** Covalent attachment of amphotericin B and fluorescein isothiocyanate to CNT. Multi-walled carbon nanotubes are treated with acids to generate carboxylic groups, subsequently modified with a mono-protected diaminotriethylene glycol. The nanotubes are then subjected to 1,3-dipolar cycloaddition. Selective cleavage of the orthogonal protecting groups allows the introduction of the fluorescein moiety and amphotericin B. **(b)** Epifluorescence microscopy image of *f*-CNT inside Jurkat cells. Bright-field image (left) and fluorescent image (right) after internalisation of *f*-CNT incubates for 16 h at 37 °C. Jurkat cells have an average diameter of 10 μm.

Drug delivery with carbon nanotubes

The search for new and effective drug delivery systems is a fundamental issue of continuous interest [39]. A drug delivery system is generally designed to improve the pharmacological and therapeutic profile of a drug molecule [40]. The ability of *f*-CNT to penetrate into the cells offers the potential of using *f*-CNT as vehicles for the delivery of small drug molecules [25•,26•]. However, the use of *f*-CNT for the delivery of anticancer, antibacterial or antiviral agents has not yet been fully ascertained. The development of delivery systems able to carry one or more therapeutic agents with recognition capacity, optical signals for imaging and/or specific targeting is of fundamental advantage, for example in the treatment of cancer and different types of infectious diseases [41]. For this purpose, we have developed a new strategy for the multiple functionalisation of CNT with different types of molecules (Figure 4a) [42]. A fluorescent probe for tracking the cellular uptake of the material and an antibiotic moiety as the active molecule were covalently linked to CNT. MWNT were functionalised with amphotericin B and fluorescein.

The antibiotic linked to the nanotubes was easily internalised into mammalian cells without toxic effects in comparison with the antibiotic incubated alone (Figure 4b). In addition, amphotericin B bound to CNT preserved its high antifungal activity against a broad range of pathogens, including *Candida albicans*, *Cryptococcus neoformans* and *Candida parapsilosis*.

In an alternative approach by a different group, SWNT have been functionalised with substituted carborane cages to develop a new delivery system for an efficient boron neutron capture therapy [43]. These types of water-soluble CNT were aimed at the treatment of cancer cells. Indeed, these studies showed that some specific tissues contained carborane following intravenous administration of the CNT conjugate and, more interestingly, that carborane was concentrated mainly at the tumour site. Another class of carbon nanomaterials similar to CNT have also been used for drug delivery [44]. Single-walled carbon nanohorns are nanostructured spherical aggregates of graphitic tubes. Murakami *et al.* loaded these tubes with dexamethasone and studied the binding and release of the drug. They found that dexamethasone could be adsorbed in large amounts onto oxidised nanohorns and maintains its biological integrity after being liberated. This was confirmed by activation of glucocorticoid response in mouse bone marrow cells and induction of alkaline phosphatase in mouse osteoblasts.

In view of these results, *f*-CNT represent a new, emerging class of delivery systems for the transport and translocation of drug molecules into different types of mammalian cells. Although these CNT conjugates displayed no cytotoxicity *in vitro*, for further development, it will be important to assess their metabolism, biodistribution and clearance from the body.

Conclusions

Organic functionalisation has opened new horizons in the study of the biological properties of CNT. First of all, the biocompatibility of carbon cylinders has been ascertained. Because pristine CNT are highly toxic, mainly due to their insolubility, it was of fundamental importance to verify the solubility of *f*-CNT in physiological media. Secondly, properly functionalised CNT seem to have a high propensity to cross cell membranes. In addition, CNT can be charged with biologically active moieties, which can then be delivered to the cell cytoplasm or nucleus. The chemistry of CNT offers the possibility of introducing more than one function on the same tube, so that targeting molecules, contrast agents, drugs, or reporter molecules can be used at the same time. Though it is still too early to establish CNT for clinical use, these novel carriers are undoubtedly interesting and deserve further investigation.

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References and recommended reading

Papers of particular interest, published within the annual period of review, have been highlighted as:

- • of special interest
- •• of outstanding interest

1. Bacon R: **Growth, structure, and properties of graphite whiskers**. *J Appl Phys* 1960, **31**:284-290.

2. Oberlin A, Endo M, Koyama T: **Filamentous growth of carbon though benzene decomposition**. *J Cryst Growth* 1976, **32**:335-349.

3. Iijima S: **Helical microtubules of graphitic carbon**. *Nature* 1991, **354**:56-58.

4. Baughman RH, Zakhidov AA, de Heer WA: **Carbon nanotubes-the route toward applications**. *Science* 2002, **297**:787-792.

5. Ericson LM, Fan H, Peng H, Davis VA, Zhou W, Sulpizio J, Wang Y, Booker R, Vavro J, Guthy C *et al.*: **Macroscopic, neat, single-walled carbon nanotube fibers**. *Science* 2004, **305**:1447-1450.

6. Milne WI, Teo KBK, Amaratunga GAJ, Legagneux P, Gangloff L, Schnell JP, Semet V, Thien Binh V, Groening O: **Carbon nanotubes as field emission sources**. *J Mater Chem* 2004, **14**:933-943.

7. • Martin CR, Kohli P: **The emerging field of nanotube biotechnology**. *Nat Rev Drug Discov* 2003, **2**:29-37.
This paper summarizes the current efforts toward the development of nanotube structures for biomedical and biotechnological applications.

8. Bianco A, Prato M: **Can carbon nanotubes be considered useful tools for biological applications?** *Adv Mater* 2003, **15**:1765-1768.

9. • Shi Kam NW, Dai H: **Carbon Nanotubes as Intracellular Protein Transporters: Generality and Biological Functionality**. *J Am Chem Soc* 2005, **127**:6021-6026.
This article describes one of the possible mechanisms of internalisation of protein-functionalised carbon nanotubes into cells.

10. Iijima S, Ichihashi T: **Single-shell carbon nanotubes of 1-nm diameter**. *Nature* 1993, **363**:603-605.

11. Bethune DS, Klang CH, de Vries MS, Gorman G, Savoy R, Vazquez J, Beyers R: **Cobalt-catalysed growth of carbon nanotubes with single-atomic-layer walls**. *Nature* 1993, **363**:605-607.

12. Colvin VL: **The potential environmental impact of engineered nanomaterials**. *Nat Biotechnol* 2003, **21**:1166-1170.

13. • Lin Y, Taylor S, Li H, Fernando KA, Qu L, Wang W, Gu L, Zhou B, Sun YP: **Advances toward bioapplications of carbon nanotubes**. *J Mater Chem* 2004, **14**:527-541.
The authors present a comprehensive overview of covalent and non covalent functionalisation of carbon nanotubes toward biological applications.

14. Bianco A, Kostarelos K, Partidos CD, Prato M: **Biomedical applications of functionalised carbon nanotubes**. *Chem Commun* 2005:571-577.

15. Kostarelos K, Lacerda L, Partidos CD, Prato M, Bianco A: **Carbon nanotube-mediated delivery of peptides and genes to cells: translating nanobiotechnology to therapeutics**. *J Drug Deliv Sci Technol* 2005, **15**:41-47.

16. Bianco A: **Carbon nanotubes for the delivery of therapeutic molecules**. *Expt Opin Drug Deliv* 2004, **1**:57-65.

17. Liu J, Rinzler AG, Dai H, Hafner JH, Bradley RK, Boul PJ, Lu A, Iverson T, Shelimov K, Huffman CB *et al.*: **Fullerene pipes**. *Science* 1998, **280**:1253-1256.

18. Tasis D, Tagmatarchis N, Georgakilas V, Prato M: **Soluble carbon nanotubes**. *Chemistry* 2003, **9**:4000-4008.

19. Dyke CA, Tour JM: **Overcoming the insolubility of carbon nanotubes through high degrees of sidewall functionalization**. *Chemistry* 2004, **10**:812-817.

20. Georgakilas V, Kordatos K, Prato M, Guldi DM, Holzinger M, Hirsch A: **Organic functionalization of carbon nanotubes**. *J Am Chem Soc* 2002, **124**:760-761.

21. • Georgakilas V, Tagmatarchis N, Pantarotto D, Bianco A, Briand JP, Prato M: **Amino acid functionalisation of water soluble carbon nanotubes**. *Chem Commun (Camb)* 2002: 3050-3051.
This paper reports the preparation of amine-functionalised water-soluble SW and MWNT.

22. Pantarotto D, Partidos CD, Graff R, Hoebeke J, Briand JP, Prato M, Bianco A: **Synthesis, Structural characterization and immunological properties of carbon nanotubes functionalized with peptides**. *J Am Chem Soc* 2003, **125**:6160-6164.

23. Pantarotto D, Partidos CD, Hoebeke J, Brown F, Kramer E, Briand JP, Muller S, Prato M, Bianco A: **Immunization with peptide-functionalized carbon nanotubes enhances virus-specific neutralizing antibody responses**. *Chem Biol* 2003, **10**:961-966.

24. Salvador-Morales C, Flahaut E, Sim E, Sloan J, Green MLH, Sim RB: **Complement activation and protein adsorption by carbon nanotubes**. *Mol Immunol* 2005, in press, doi:10.1016/j.molimm.2005.02.006.

25. • Pantarotto D, Briand JP, Prato M, Bianco A: **Translocation of bioactive peptides across cell membranes by carbon nanotubes**. *Chem Commun (Camb)* 2004:16-17.
This paper demonstrates the cellular uptake of functionalised carbon nanotubes.

26. • Shi Kam NW, Jessop TC, Wender PA, Dai H: **Nanotube molecular transporters: internalization of carbon nanotube-protein conjugates into mammalian cells**. *J Am Chem Soc* 2004, **126**:6850-6851.
This paper elucidates one of the possible mechanisms of carbon nanotube internalisation.

27. Pantarotto D, Singh R, McCarthy D, Erhardt M, Briand JP, Prato M, Kostarelos K, Bianco A: **Functionalised carbon nanotubes for plasmid DNA gene delivery**. *Angew Chem Int Ed Engl* 2004, **43**:5242-5246.

28. Lopez CF, Nielsen SO, Moore PB, Klein ML: **Understanding nature's design for a nanosyringe**. *Proc Natl Acad Sci USA* 2004, **101**:4431-4434.

29. Cai D, Mataraza JM, Qin ZH, Huang Z, Huang J, Chiles TC, Carnahan D, Kempa K, Ren Z: **Highly efficient molecular delivery into mammalian cells using carbon nanotube spearing**. *Nat Methods* 2005, **2**:449-454.

30. Cherukuri P, Bachilo SM, Litovsky SH, Weisman RB: **Near-infrared fluorescence microscopy of single-walled carbon nanotubes in phagocitic cells**. *J Am Chem Soc* 2004, **126**:15638-15639.

31. Luo D: **A new solution for improving gene delivery**. *Trends Biotechnol* 2004, **22**:101-103.

32. Schmidt-Wolf GD, Schmidt-Wolf IG: **Non-viral and hybrid vectors in human gene therapy: an update**. *Trends Mol Med* 2003, **9**:67-72.

33. Chaudhuri A (Ed.): **Special issue: Cationic transfection lipids**. *Curr Med Chem* 2003, **10**:1185-1315.

34. Dean DA, Strong DD, Zimmer WE: **Nuclear entry of nonviral vectors**. *Gene Ther* 2005, **12**:881-890.

35. Singh R, Pantarotto D, McCarthy D, Chaloin O, Hoebeke J, Partidos CD, Briand JP, Prato M, Bianco A, Kostarelos K: **Binding and condensation of plasmid DNA onto functionalized carbon nanotubes: towards the construction of nanotube-based gene delivery vectors**. *J Am Chem Soc* 2005, **127**:4388-4396.

36. Liu Y, Wu DC, Zhang WD, Jiang X, He CB, Chung TS, Goh SH, Leong KW: **Polyethylenimine-grafted multiwalled carbon nanotubes for secure noncovalent immobilization and efficient delivery of DNA**. *Angew Chem Int Ed Engl* 2005, **44**:4782-4785.

37. Bianco A, Hoebeke J, Godefroy S, Chaloin O, Pantarotto D, Briand JP, Muller S, Prato M, Partidos CD: **Cationic carbon nanotubes bind to CpG oligodeoxynucleotides and enhance their immunostimulatory properties**. *J Am Chem Soc* 2005, **127**:58-59.

38. Lu Q, Moore JM, Huang G, Mount AS, Rao AM, Larcom LL, Ke PC: **RNA polymer translocation with single-walled carbon nanotubes**. *Nano Lett* 2004, **4**:2473-2477.

39. Allen TM, Cullis PR: **Drug delivery systems: entering the mainstream**. *Science* 2004, **303**:1818-1822.

40. Kostarelos K: **Rational design and engineering of delivery systems for therapeutics: biomedical exercises in colloid and surface science**. *Adv Colloid Interface Sci* 2003, **106**:147-168.

41. Ferrari M: **Cancer nanotechnology: opportunities and challenges**. *Nat Rev Cancer* 2005, **5**:161-171.

42. Wu W, Wieckowski S, Pastorin G, Klumpp C, Benincasa M, Briand JP, Gennaro R, Prato M, Bianco A: **Targeted delivery of amphotericin B to cells using functionalised carbon nanotubes**. *Angew Chem Int Ed Engl* 2005, in press, doi: 10.1002/anie.200501613.

43. Yinghuai Z, Peng AT, Carpenter K, Maguire JA, Hosmane NS, Takagaki M: **Substituted carborane-appended water-soluble single-wall carbon nanotubes: new approach to boron neutron capture therapy drug delivery**. *J Am Chem Soc* 2005, **127**:9875-9880.

44. Murakami T, Ajima K, Miyawaki J, Yudasaka M, Iijima S, Shibe K: **Drug-loaded carbon nanohorns: adsorption and release of dexamethasone *in vitro***. *Mol Pharm* 2004:399-405.