

# Biomedical applications of functionalised carbon nanotubes

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The organic functionalisation of carbon nanotubes can improve substantially their solubility and biocompatibility profile; as a consequence, their manipulation and integration into biological systems has become possible so that functionalised carbon nanotubes hold currently strong promise as novel systems for the delivery of drugs, antigens and genes.

Carbon nanotubes (CNTs) are nanoobjects that have raised great expectations in a number of different applications, including field emission,<sup>1</sup> energy storage,<sup>2</sup> molecular electronics,<sup>3</sup> atomic force microscopy,<sup>4</sup> and many others.<sup>5</sup> The backbone of CNTs is composed solely of carbon atoms, arranged in benzene rings forming graphene sheets, rolled up to give seamless cylinders. There are two main types of CNTs, single-walled (SWCNTs) and multi-walled carbon nanotubes (MWCNTs), the latter being formed by several concentric

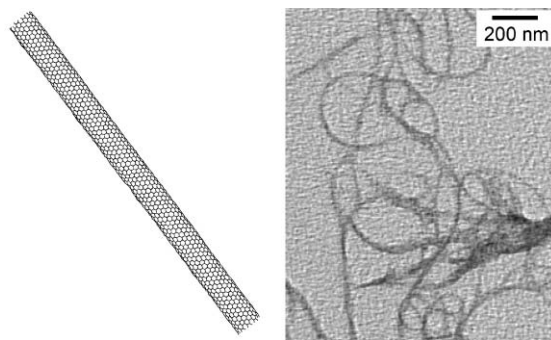


Fig. 1 Model structure of a SWCNT segment (left) and TEM image of SWCNTs (right).

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layers of rolled graphite. In particular, SWCNTs are characterised by a high ratio of length over diameter (aspect ratio), *i.e.*, they can be very long (up to several microns) and very thin (a few nm, see Fig. 1).

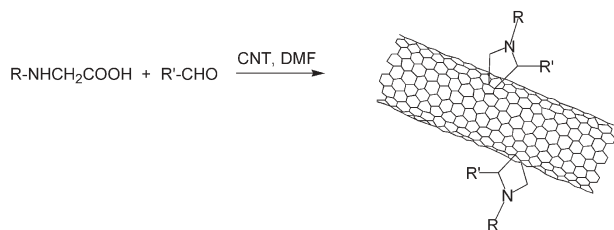
Applications of CNTs in the field of biotechnology have recently started to emerge, raising great hopes.<sup>6</sup> CNTs have been proposed as components for DNA and protein biosensors,<sup>7</sup> ion channel blockers<sup>8</sup> and as bioseparators and biocatalysts.<sup>9</sup> Concerning the biomedical applications of CNTs, their use is becoming relevant in neuroscience research and tissue engineering. They have been developed as scaffolds for neuronal and ligamentous tissue growth for regenerative interventions of the central nervous system (*e.g.* brain, spinal cord) and orthopaedic sites.<sup>10</sup> CNTs have also been used as new platforms to detect antibodies associated with human autoimmune diseases with high specificity.<sup>11</sup> This finding paves the way to the development of carbon nanotube-based diagnostic devices for the discrimination and identification of different proteins from serum samples and in the fabrication of microarray devices for proteomic analyses. In a similar context, carbon nanotubes covalently modified at their open ends with DNA and PNA (peptide nucleic acid) have led to innovative systems for hybridization of complementary DNA strands allowing for ultrasensitive DNA detection.<sup>7d,12</sup>

A major drawback of CNTs particularly relevant to their compatibility with biological systems is their complete insolubility in all types of solvents.<sup>5</sup> However, CNTs have been found to exhibit a certain degree of chemical reactivity towards many reagents, thus leading to increased solubility and processability. So far different methodologies have been reported and recently reviewed.<sup>13</sup>

The main aim of this Feature Article is to review the efforts, carried out in our laboratories, which have led us to propose CNTs as promising new systems for a variety of potential biomedical applications. In particular, the description of CNT surface functionalisation will be followed by our recent findings using functionalised CNTs (*f*-CNTs) as drug, vaccine and gene delivery systems.

## Functionalised carbon nanotubes (*f*-CNTs)

One of the most powerful methods, particularly suitable for the preparation of soluble CNTs, is the 1,3-dipolar cycloaddition of azomethine ylides.<sup>14</sup> This reaction occurs in a suspension of pristine carbon nanotubes in dimethylformamide (DMF) followed by the addition of an *N*-substituted glycine and an aldehyde (Scheme 1).



**Scheme 1** General scheme for the 1,3-dipolar cycloaddition of azomethine ylides to CNTs.

This reaction works efficiently with both SWCNTs and MWCNTs. The functionalised CNTs are now sufficiently soluble in the common organic solvents to be characterised by standard spectroscopic means (*i.e.*, FT-IR and NMR). One of the most powerful techniques for directly observing CNTs is electron microscopy. Transmission Electron Microscopy (TEM) allows observation of the functionalised single-walled carbon nanotubes (*f*-SWCNTs) as bundles of different diameter and length, while *f*-MWCNTs are visualised as single entities. Complementary techniques such as Scanning Electron Microscopy (SEM) and Atomic Force Microscopy (AFM) are also very helpful in elucidating the structure of the tubes.

The amount of functional groups around the tips and side-walls of the CNTs can be quantified using different techniques, such as absorption spectroscopy, calorimetric analyses, *etc.* In some cases, when free amino groups are present on the CNT surface, such as in **1** (Fig. 2), the Kaiser test is extremely useful for quantitative evaluations.<sup>15</sup> All these techniques have given us very similar values about the degree of functionalisation, which was found to be different for SWCNTs and MWCNTs. Whereas the former showed usually a loading of 0.3–0.5 mmol of functional groups per gram of material, MWCNTs carried about 0.5–0.9 mmol g<sup>-1</sup>.

## Coupling of amino acids and peptides to *f*-CNT

Many different functional groups can be placed at the tips and around the side-walls of CNTs. A typical example of a versatile intermediate, which can then be very easily derivatised, is compound **1** in Fig. 2. In fact, a series of amino acids, fluorescent probes and bioactive peptides have been covalently linked to CNTs through the amino groups in **1** (derivatives **2–8**, Fig. 2). In addition, *f*-CNTs **1** are highly soluble in water, which allows the formation of supramolecular complexes with biologically-relevant macromolecules and substrates, based on electrostatic interactions. Typical electron microscopy images of *f*-MWCNTs **1** obtained by deposition from a water solution are shown in Fig. 3.

We initially tested the possibility of covalently coupling bioactive moieties to **1** using an *N*-protected glycine. The resulting *f*-CNTs **2** were characterised by TEM and NMR. The <sup>1</sup>H NMR spectrum of the functionalised SWCNTs **2** confirmed the presence of the functionalised oligoethylene glycol chain and the aromatic protons of the Fmoc group. The <sup>15</sup>N-labelled nitrogen on the Gly residue was introduced to perform heteronuclear correlation experiments. The single peak measured at –315.57 ppm in deuterated acetonitrile (referenced to the external standard nitromethane) was indicative of a homogeneous distribution of the *N*-protected amino acid around the nanotube side-wall.<sup>15a</sup>

Once we ascertained the possibility of peptide synthesis utilising *f*-CNTs **1**, we embarked on a systematic study, aimed at exploiting the use of CNTs in biology. The functionalisation of the single-walled carbon nanotubes with peptides was tackled using different strategies (Scheme 2).

The fragment condensation allows linkage of the C-terminal activated carboxylic group of a fully protected peptide to the amino functions localised on the external walls of the tubes. For this purpose, we have chosen the model sequence H-Lys-Gly-Tyr-Tyr-Gly-OH. After conjugation to *f*-CNTs **1**, the peptide–CNTs was treated with acid to remove the Boc

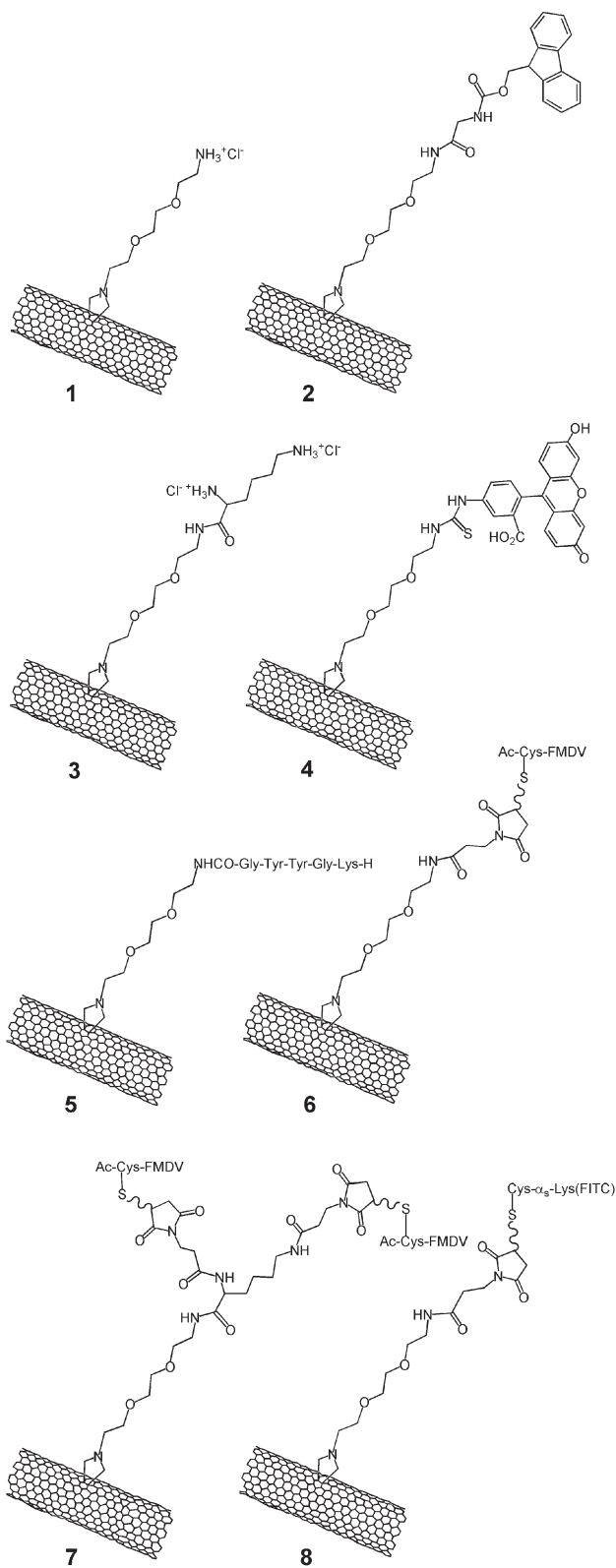


Fig. 2 Structural drawings of *f*-CNTs.

protecting groups at the N-terminus and at the lysine and tyrosine side chains, thus obtaining *f*-CNTs **5**.<sup>15b</sup> Other groups have studied the interactions of CNTs with peptides, but unfunctionalised CNTs were used in that case. The aim was to

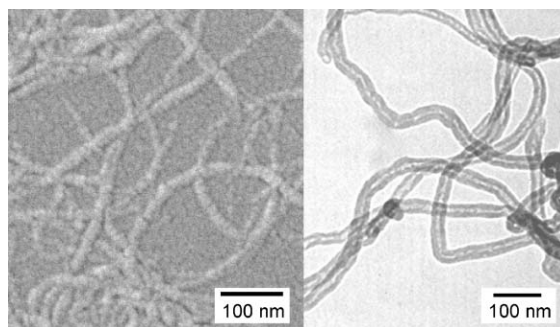
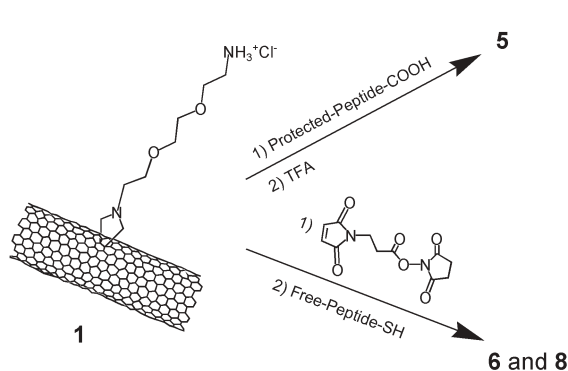


Fig. 3 Scanning electron microscopy (left) and transmission electron microscopy (right) photographs of water-soluble *f*-MWCNTs **1**.



Scheme 2 Synthesis of peptide-CNT conjugates using fragment condensation (*f*-CNTs **5**) and selective chemical ligation (*f*-CNTs **6** and **8**).

form supramolecular complexes with amphiphilic helical peptides capable of wrapping the tubes and rendering them soluble to facilitate their manipulation.<sup>16</sup> In another application, SWCNTs were used to select hydrophobic peptides from a peptide library.<sup>17</sup> Our approach of having the peptides covalently linked to CNTs *via* a covalent bond was dictated by the prospect of using CNTs for vaccine and drug delivery.

### Functionalised carbon nanotubes for drug delivery

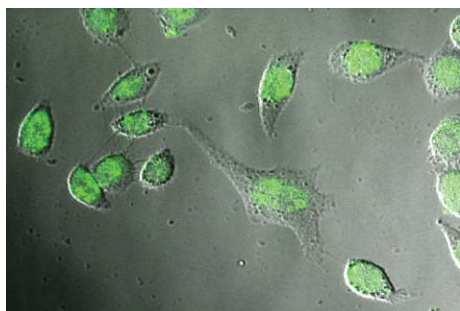
The search for new and effective drug delivery systems is rapidly expanding.<sup>18</sup> Many different delivery systems and methodologies are currently available.<sup>19</sup> They have been developed according to the different classes of bioactive molecules to be delivered (*e.g.* peptides, proteins, nucleic acids and small organic molecules) and the characteristics of the target tissues. Liposomes, emulsions, cationic polymers, micro- and nanoparticles are the most commonly studied vehicles.<sup>20</sup> A drug delivery system is generally designed to improve the pharmacological and therapeutic profile of a drug molecule.<sup>18,21</sup> Problems associated with the administration of free drugs, such as limited solubility, poor biodistribution, lack of selectivity, unfavourable pharmacokinetics, healthy tissue damage, can be overcome and/or ameliorated by the use of a drug delivery system. However, it is impossible to conceive and engineer a universal system. Recently, new approaches are emerging in the field of drug delivery, mainly due to significant advances in nanotechnology and nanofabrication.<sup>6b,22</sup> The future generations of drug delivery devices may well include



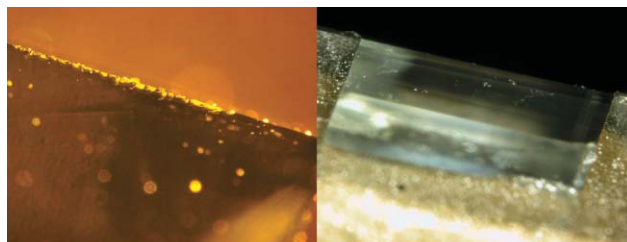
for example microchip-controlled release drug reservoirs,<sup>19a,23</sup> lipid, peptide or silica nanotubes and carbon nanotubes.<sup>6b-d</sup>

The aqueous solubility and cationic surface character of *f*-CNTs **1** render them potentially novel delivery vehicles. In this context, we initially demonstrated the capability of carbon nanotubes to cross the plasma membrane and distribute throughout the cellular compartments. We prepared two different fluorescently labelled carbon nanotubes, by attachment of either fluorescein isothiocyanate (FITC) directly to the amino functions of the soluble carbon nanotubes or a fluorescent peptide using the chemical ligation approach (for their molecular structures see conjugate **4** and **8** in Fig. 2).<sup>24</sup> The selective chemical ligation permits the binding of a fully deprotected and purified peptide *via* the specific reaction of a cysteine thiol group, of the peptide sequence, to a maleimido moiety introduced on the *f*-CNTs **1** (Scheme 2).<sup>15b</sup> Such a peptide belongs to the  $\alpha$  subunit of the G<sub>s</sub> protein and corresponds to the sequence 384–394, modified with a cysteine at the C-terminus for conjugation to the nanotubes and a lysine at the N-terminal part bearing the label FITC. This peptide mimics the effect of G<sub>s</sub> protein by increasing the agonist affinity for the  $\beta$ -adrenergic receptor. It has been shown that the cellular uptake of the free peptide is extremely poor. To assess the capability of the carbon nanotubes to penetrate HeLa cells, both conjugates were incubated with the cells and analysed with fluorescence microscopy. Fig. 4 shows the fluorescent carbon nanotubes localised intracellularly. While the *f*-CNT conjugate **4** with the FITC alone mainly distributed into the cytoplasm, slowly moving towards the nucleus, the peptide–CNTs **8** rapidly translocated to the cell nucleus.

The same results were obtained using other types of cells including human and murine fibroblasts and keratinocytes. In an alternative approach, ammonium functionalised MWCNTs, devoid of any fluorescent probe, were directly visualised into cells using TEM.<sup>25</sup> After incubation of the cells with *f*-CNTs, followed by washings, staining and embedding into a polymer matrix, slices of 90 nm thickness were cut with different microtome knives for imaging under TEM. We initially used a glass knife and immediately realised that the edge of the tool was completely damaged (Fig. 5, left). For this reason, we sectioned the polymer containing the CNTs by using a diamond knife.<sup>26</sup> A close inspection through the optical microscope of the diamond edge allowed us to conclude that the device was still intact (Fig. 5, right).



**Fig. 4** Epifluorescence image of HeLa cells incubated at 37 °C for 1 hour with 5  $\mu$ M concentration of *f*-CNTs **8**.



**Fig. 5** Ultramicrotome knives: images of the glass (left), and diamond tool (right) taken with an optical microscope.

The *f*-MWCNTs **1** were clearly visible in the TEM images throughout the cellular compartments, distributed in the cytoplasm and the nucleus. Although the elucidation of the mechanism of entry requires further investigations, we excluded endocytosis. This is because inhibitors of endosome-mediated translocation such as sodium azide and 2,4-dinitrophenol, and the decrease of the incubation temperature to 4 °C, did not prevent cellular uptake of the different *f*-CNTs.<sup>24</sup> In addition, the TEM images revealed the tubes crossing the cell membrane as nanoneedles without any perturbation or disruption of the membrane.<sup>25</sup>

Cell viability after treatment with *f*-CNTs is an important issue that we are currently addressing. Cytotoxicity of carbon nanotubes with no functionalisation has been recently studied by different research groups.<sup>27</sup> The main causes of toxicity are the presence of residual metal catalyst and the insolubility of the material. Functionalised CNTs are instead highly soluble in aqueous biological media and exhibit notably reduced cellular toxicity *in vitro*. Cell viability was studied using flow cytometry by treating HeLa cell cultures with increasing doses of *f*-SWCNTs and *f*-MWCNTs and for long incubation times. We found that 50% of the cells died after a 6 hour incubation period with 5–10 mg ml<sup>-1</sup> of nanotube solution, a concentration considered excessively high.<sup>25</sup> We also observed that FITC functionalised carbon nanotubes were more toxic than those with the free ammonium group.<sup>24</sup> Comparable results have been very recently reported by Wender and Dai who demonstrated that carbon nanotubes can drive surface-bound proteins into cells.<sup>28</sup> CNTs were initially functionalised by oxidation and sonication. The carboxylic groups generated after this treatment were derivatised either with FITC or with biotin. CNTs with biotin were subsequently complexed with fluorescent streptavidin. Both types of fluorescent–CNT conjugates were incubated with HL60 cells showing membrane translocation with reduced toxicity using confocal microscopy and flow cytometry. In addition, the strong streptavidin–CNT supramolecular assembly was demonstrated to be taken up by cells *via* an endocytosis mechanism. Even though this proposed mechanism is in contrast to the internalisation process we have observed with our conjugates, in the case of the Stanford group, endocytosis might likely be induced by streptavidin. However, with the currently available data it is difficult to propose a general mechanism of cellular uptake of CNTs. In general, these results could be considered as extremely promising, although improvement and complementary studies on *f*-CNT metabolism, distribution and elimination *in vitro* and *in vivo* are essential before any type of application.

In summary, since functionalised carbon nanotubes penetrate easily into cells with reduced toxicity, they can be considered very interesting and innovative carriers for drug delivery. In view of the different mechanisms of cellular internalisation and the observation that intracellular distribution depends on the functional groups (FITC, peptides, proteins), it can be argued that the various cellular compartments can be selectively targeted.

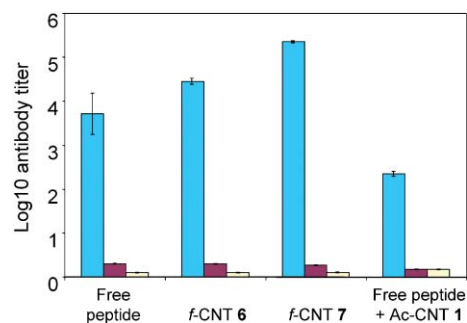
## Functionalised carbon nanotubes for presentation and delivery of antigens

In principle, *f*-CNTs can be considered ideal vector systems for peptide antigens. In fact, due to their high aspect ratio, *f*-CNTs offer a potentially high loading capacity for cargo molecules. In this context, we were interested in linking synthetic peptides to CNTs to study their immunogenic properties. We pursued peptide coupling to CNTs using selective chemical ligation (Scheme 2).<sup>15b</sup> As a model antigen we used a B cell epitope from the foot-and-mouth disease virus (FMDV). This peptide corresponds to the sequence 141–159 (GSGVRGDFGSLA-PRVARQL) of the VP1 protein of the virus, modified at the N-terminal part with an acetylated cysteine. This peptide was selected because it represents a virus neutralising and protective epitope. The peptide was linked to the *f*-CNTs **1** either as a mono-conjugate or as a bis-conjugate (for their molecular structures see conjugate **6** and **7** in Fig. 2). In the first case, *f*-CNTs **1** were initially derivatised with a maleimido group using *N*-succinimidyl-3-maleimidopropionate in DMF. The tubes were further modified by the selective reaction of the FMDV peptide cysteine residue to the maleimide moiety. This reaction was performed in water and the excess peptide was trapped by addition of a scavenger resin, which enabled blocking and elimination of the peptide. The course of the reaction was followed by HPLC by detecting the decrease of the peptide concentration during the conjugation step. Indeed, the formation of *f*-CNTs **6** could not be detected because the molecule was too big to pass through the HPLC column and it was retained in the precolumn system. The final conjugate was recovered as a brown solid material after lyophilisation. For the preparation of the bis-conjugate **7**, the tubes **1** were first derivatised with a lysine residue subsequently modified with two maleimido groups at the main and side-chain amino functions.<sup>29</sup> Following this procedure, it was possible to insert two FMDV peptides. The purification and isolation steps were the same as for the mono-conjugate. The peptide–CNT systems were characterised by TEM and NMR. In particular, 2D NMR experiments allowed for assignment of the amino acid sequence and analysis of the secondary structure adopted by the peptides onto the nanotubes. The chemical shift dispersion, the intensity and the position of the NOE signals for the peptide mono- and bis-conjugates were identical to those of the FMDV free in solution. This finding suggested that the peptide covalently bound to the carbon nanotube or free in solution displayed a similar conformation.

We first studied the antigenic properties of peptide–CNTs **6** and **7**. Antigenicity is the capacity of an antigen to be specifically recognised by an antibody. This characteristic can be evaluated using enzyme-linked immunosorbent assays

(ELISA) and surface plasmon resonance (SPR).<sup>15b,29</sup> The conjugates **6** and **7**, the free peptide and the acetylated *f*-CNTs **1** were coated on the ELISA plates and incubated with specific monoclonal and polyclonal antibodies. The peptide–CNT conjugates were recognised by the antibodies equally well as the free peptide while the functionalised carbon nanotubes, devoid of the peptide moiety, were not recognised. Therefore, we could conclude that the secondary structure of the nanotube-linked peptide, necessary for the spatial interaction with the antibody, is properly presented by the CNTs. This was further confirmed by the SPR experiments. By using the SPR technique it was possible to study the antigen–antibody interactions in real time. The specific anti-FMDV 141–159 peptide monoclonal antibody was fixed to a gold chip *via* a second antibody. Then a solution of free FMDV peptide, or acetylated *f*-CNTs **1**, or *f*-CNTs **6** and **7** was passed over the immobilised antibody to measure the increase in mass due to the interaction between the different entities. CNTs without any bound peptide did not react with the antibody, whereas free peptide, mono-conjugate **6**, and bis-conjugate **7**, interacted with increasing mass. These results suggested again that the peptides linked to CNTs retained their structural characteristics for recognition by the specific antibody.

We also studied the immunogenic properties of these molecules.<sup>29</sup> Immunogenicity is the ability of an antigen to elicit an immune response. Four groups of mice were immunised with the free FMDV peptide, the mono-conjugate *f*-CNTs **6**, the bis-conjugate *f*-CNTs **7** and a mixture of the free peptide with *f*-CNTs **1**, respectively. Since the FMDV B-cell epitope does not contain a T-cell epitope, we co-immunised it with ovalbumin, which provided T-cell help. Immunisation of mice with **6** and **7** clearly enhanced anti-FMDV peptide antibody responses as compared to the free peptide and to the mixture of the free peptide with *f*-CNTs **1** (Fig. 6). The antibodies were peptide-specific and were not directed to the peptide–CNT linker. From these studies it was evident that it is necessary to conjugate the peptides to the carbon nanotubes *via* a stable covalent bond, since the simple mixture of the two components did not elicit high antibody titers. Most importantly, no antibodies against *f*-CNTs **1**, devoid of peptide moiety, were detected. The lack of immune response to CNTs



**Fig. 6** Anti-peptide antibody responses following immunisation with peptide and peptide–CNT conjugates. Serum samples were screened by ELISA for the presence of antibodies using FMDV 141–159 peptide conjugated to BSA (cyan bar), control peptide conjugated to BSA (magenta bar) or CNTs **1** functionalised with maleimido group without peptide (white bar) as solid-phase antigens.

is important in view of epitopic suppression that can be observed after several administrations of peptide antigens coupled to carrier proteins. Finally, we demonstrated the ability of anti-peptide antibodies to neutralise the virus. Surprisingly, *f*-CNTs **6** elicited significantly higher virus neutralising antibody responses than those induced by *f*-CNTs **7** although the latter induced higher antibody titers. Probably these antibodies lack the correct specificity as the two copies of the FMDV peptide bound to the CNTs interacted *in vivo* and adopted a conformation different from that displayed by the native epitope on the virus. These results are in a good agreement with previous observations showing the limitation of multiple presentation of epitopes to elicit antibodies cross-reactive with the native protein.<sup>30</sup>

In conclusion, functionalised carbon nanotubes can be considered as an interesting and promising carrier system for the delivery of candidate vaccine antigens based for example on peptides and proteins.

### Functionalised carbon nanotubes for gene delivery

Gene therapy is one of the most promising approaches to treat a variety of different diseases, such as cancer and genetic disorders.<sup>31</sup> Gene delivery is based on the development and use of viral and non-viral vector systems. However great concerns have recently been raised on virus-based genetic intervention strategies, since these vectors can induce undesired immune responses, inflammations and oncogenic side-effects.<sup>32</sup> Non-viral vectors have been explored as alternatives that can guarantee a higher degree of safety. Non-viral vectors include nucleic acid conjugates with liposomes, cationic lipids, polymers, micro- and nanoparticles and many others.<sup>33</sup> However, serious limitations are also presented by non-viral gene vector systems, primarily related to the poor pharmacokinetic profiles of the administered oligonucleotide and plasmid DNA conjugates, and the low levels of gene expression obtained.<sup>34</sup> Therefore, research efforts focus on the development of effective delivery systems for gene-encoding nucleic acids (DNA, RNA, ODN, *etc.*).<sup>35</sup>

With the aim of developing different alternative gene carrier systems, we examined the potential of functionalised carbon nanotubes. We reasoned that the macromolecular and cationic nature of the functionalised carbon nanotubes (*e.g.*, *f*-CNTs **1**) should help to form supramolecular complexes with plasmid DNA. Other groups have studied the interaction of carbon nanotubes with single stranded DNA (ssDNA), aiming to increase the solubility of nanotubes in aqueous solution and to reduce their polydispersity. For example, it has been demonstrated that SWCNTs have high affinity for ssDNA, presumably by hydrophobic interactions.<sup>36</sup> In addition, CNTs have been functionalised covalently with DNA, allowing the formation of supramolecular nanotube-based structures by DNA–DNA interactions.<sup>12,37</sup>

To explore the potential of *f*-CNTs as gene transfer vectors, we carried out an initial study to evaluate the ability of *f*-CNTs **1** to condense plasmid DNA. We decided to form a complex between pCMV- $\beta$ gal, which expresses the  $\beta$ -galactosidase protein, and *f*-CNTs **1**, solely due to electrostatic interactions. Different charge ratios between the two components were

studied. The observation of the ensuing supramolecular assemblies using TEM indicated that nanotube–DNA complexes were formed (Fig. 7). The *f*-SWCNTs were presented in bundles of different diameters on which the plasmid DNA was condensed by forming toroidal clusters, or globular and supercoiled structures. This observation was extremely encouraging for the subsequent planning of gene delivery and expression experiments. Indeed, we were able to obtain a clear effect by using the DNA complexes with *f*-CNTs **1** to carry the  $\beta$ -gal gene inside the cells, by monitoring the expression of  $\beta$ -galactosidase. Improved levels of gene expression were obtained for the *f*-CNT **1** : DNA complex in the range of positive:negative charge ratios between 2:1 to 6:1. Gene expression offered by the complexes between plasmid DNA with *f*-CNTs was 5 to 10 times higher than that of DNA alone.<sup>25</sup> Preliminary comparative gene expression data between *f*-CNT : DNA and commercially available lipid : DNA delivery systems showed that our first generation *f*-CNT-based gene delivery system is less efficient for *in vitro* transfection than the lipid : DNA system. However, we are confident that there is a lot of room for further improvement of the carbon nanotube system for gene delivery.

Therefore, these promising results open the possibility to exploit functionalised carbon nanotubes in gene therapy and genetic vaccination.

### Conclusions and perspectives

Carbon nanotubes are among the novel emerging technologies with potential application to drug, vaccine and gene delivery. Functionalisation of their surface can result in highly soluble materials, which can be further derivatised with active molecules, making them compatible with biological systems. Therefore, many biomedical applications can be envisaged. Surface functionalization enables adsorption or attachment of various molecules or antigens, which subsequently can be targeted to the desired cell population for immune recognition or a therapeutic effect. Although safety issues have still to be thoroughly examined, there are potentially practical benefits in developing novel vectors for nanomedicinal applications. On the other hand, progress in carbon nanotube technology may well lead to better insights into biological and physical chemistry processes. This will make it possible to find compounds more compatible with carbon nanotube technology and to facilitate more effective use of nanotubes as

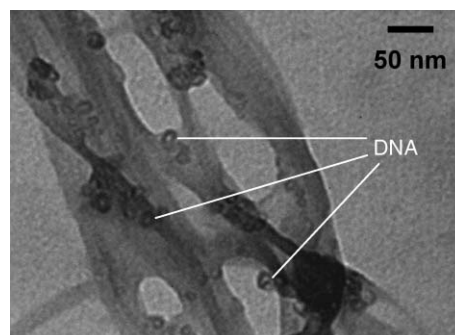


Fig. 7 TEM image *f*-CNTs **1** : DNA complexes.



delivery systems in therapeutic, preventive and diagnostic nanomedicine applications.

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## Notes and references

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