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Carbon nanotubes for the delivery of therapeutic molecules

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Functionalised carbon nanotubes (*f*-CNTs) are emerging as new tools in the field of nanobiotechnology and nanomedicine. This is because they can be easily manipulated and modified by encapsulation with biopolymers or by covalent linking of solubilising groups to the external walls and tips. The possibility of incorporating *f*-CNTs into biological systems has opened the way to the exploration of their potential applications in biology and medicinal chemistry. Within the different fields of applications (i.e., biosensors, composite materials, molecular electronics), one use of CNTs is as new carrier systems for the delivery of therapeutic molecules. Research discussed in this review is focused on recent advances in the development of CNT technology for the delivery of drugs, antigens and genes.

Keywords: antigens, carbon nanotubes, drug delivery, gene transfer, peptides, plasmid DNA, vaccine delivery, vaccines

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1. Introduction

Efforts towards the development of new methodologies for the delivery of therapeutic molecules have remarkably increased over the past few years [1-3]. There is a continuous demand for novel delivery systems that are capable of protecting, transporting and releasing active molecules (i.e., drugs, antigens, proteins, enzymes, nucleic acids) to specific sites of action.

Several routes can be employed to deliver drugs, and a series of clever devices have been engineered. Particularly, there is an emerging field of research related to the conception and fabrication of small-scale systems for drug delivery [4]. These systems include spherical micro- and nanoparticles, liposomes and micelles, dendrimers, polymers and micro- and nanotubes [5-12]. Their development has been made possible by rapid advances in micro- and nanotechnologies. Indeed, increased knowledge in the field of nanotechnology and nanofabrication has had an immediate impact on the field of delivery [13]. A drug delivery system is generally designed to improve the pharmacological and therapeutic properties of conventional drugs [1]. Problems associated with the administration of free drugs, such as limited solubility, poor biodistribution, lack of selectivity, unfavourable pharmacokinetics and 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.

Besides all established tools for drug delivery, researchers are already working on the next generation of devices [14]. New approaches are being investigated, including, for example, controlled-release drug reservoirs using microchips or nanotubes [2,3]. Nanotube technology is a field of research in full expansion. Among the different types of nanotubes, carbon nanotubes (CNTs) are very promising and gaining a lot of interest. In addition, the exploration of CNTs as a delivery system is certainly a new challenge [15,16].

The aim of this article is to describe the potential of CNTs for the delivery of therapeutic molecules, including drugs, antigens and genes. After a brief introduction to CNTs and the methodologies to render them biocompatible, the capacity

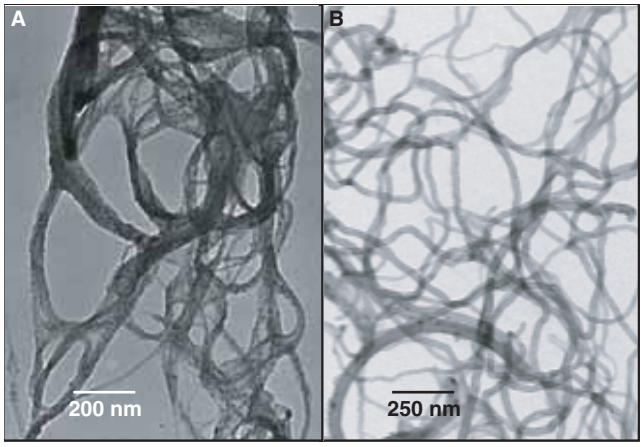

Figure 1. Transmission electron microscopy photographs of pristine single-walled (A) and multi-walled (B) carbon nanotubes.

of encapsulation and movement of molecules inside and towards their cavity will be discussed. This method of delivery will be complemented by the description of the capacity of functionalised *(f)*-CNTs with bioactive molecules at their external surface to deliver their cargo into the cells for immune intervention and potential therapeutic treatments.

2. Carbon nanotubes

CNTs were discovered by Bacon in the late 1950s, but they were not fully appreciated at that time [17]. In the 1970s, Endo and co-workers again observed the formation of CNTs [18]. However, it was only in 1991 that CNTs started to be re-investigated and proposed as an interesting material due to their structural properties [19,20]. These molecules, which are exclusively composed of carbon atoms, belong to the family of fullerenes, the third allotropic form of carbon along with graphite and diamond. CNTs consist of graphite sheets rolled up into a tubular form. CNTs can be obtained as single-walled nanotubes (SWNTs), characterised by the presence of one layer of cylinder graphene, or multi-walled nanotubes (MWNTs), made-up of several concentric graphene sheets. CNTs are objects of nanometric dimensions. Most commonly, SWNTs have a diameter of 0.4 – 3.0 nm and a length in the range of 20 – 1000 nm, whereas MWNTs are larger objects, with a diameter in the range of 1.4 –100 nm and a length of 1 – 50 µm (Figure 1). Several methods for the production of both types of tubes and the modulation of their dimensions are available in the literature [21].

At present, CNTs are largely exploited in materials science for their mechanical, electronic, optic and magnetic properties [22]. They are also attracting the interest of several research groups in the field of biotechnological, biological and biomedical sciences [23]. The main obstacle in the utilisation of CNTs in biology and medicinal chemistry is their lack of solubility in any type of solvent. Indeed, CNTs can be stabilised in organic solvents at very low concentration, whereas they are completely insoluble in aqueous solutions. In order to incorporate them into the physiological conditions required for biological studies, strategies of stabilisation or dispersion in solution have been investigated. Two main methodologies have been devised: non-covalent functionalisation; and covalent functionalisation. Both approaches give rise to soluble conjugates, in which one component is constituted by CNTs and the counterpart is, for example, a biopolymer (peptide, protein, DNA). As a consequence, applications of CNTs as vectors for the development of a new drug delivery system can be envisaged. However, there are advantages and drawbacks related to the two strategies of solubilisation, which will be discussed in the following paragraphs.

2.1 Non-covalent functionalisation of carbon nanotubes

Pristine CNTs can be taken into solution by coating them with polymers. It has been shown that poly(vinylpyrrolidone) [24], poly(*m*-phenylenevinylene) [25], poly(styrenesulfonate) [24] and surfactants [26-28] are able to interact with the nanotubes and solubilise them. Supramolecular inclusion of CNTs can be exerted by polysaccharides [29,30], cyclodextrins [31,32], soy oil [33], oligonucleotides [34,35] and peptides [36,37]. The main mechanism involved in the non-covalent functionalisation of CNTs is either self-assembly due to hydrophobic effect, as for surfactants, or π-stacking, as for most (bio)polymers. The capacity of the biopolymers to solubilise CNTs is of great importance, as CNTs can be rendered more biocompatible. In another approach, it was shown that it is possible to exploit the hydrophobic and van der Waals interactions between polyaromatic molecules possessing functional groups and the external aromatic surface of the CNT, to bring the latter into solution. In this context, an activated carboxy-pyrene derivative was adsorbed onto the CNT and subsequently modified with proteins [38]. The complex between the CNT and streptavidin or ferritin represents an elegant example of assembly in which CNTs act as a support for the non-covalent immobilisation of biomolecules for the development of new biosensor devices and carrier systems [38]. Similarly, CNTs were decorated onto the external walls with peptide antigens, thus creating a new platform for the specific detection of antibodies associated with human autoimmune diseases [39]. Nevertheless, the non-covalent functionalisation of CNTs contains an intrinsic limitation for delivery applications. Although the complex between a bioactive molecule (i.e., proteins and/or oligonucleotides) and a CNT is promptly formed, once this complex is internalised into a cell, a tissue or an organ, and the cargo molecule is released, the free nanotube will immediately aggregate and/or precipitate with a likely toxic effect [40-43]. Concerns about the toxicity of CNTs have recently been taken into account [41-43]. It has been demonstrated that the toxic effects on cells and organs are due, in part, to the insolubility of CNTs and the presence of catalyst particles, which are necessary to produce CNTs.

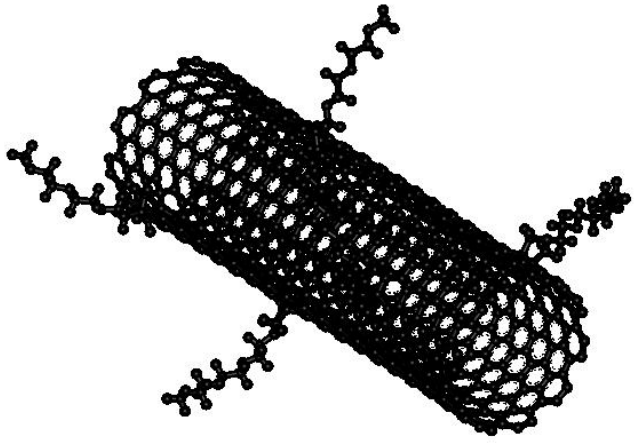

Figure 2. Molecular model of a fragment of a functionalised carbon nanotube obtained via 1,3-dipolar cycloaddition.

2.2 Covalent functionalisation of carbon nanotubes

The covalent modification of the external surface of CNTs using organic reactions is an alternative and efficient approach to obtain a soluble material in a wide range of solvents [23]. Many reactions have been used to link functional groups to the external walls and tips of CNTs. It is beyond the scope of this article to describe all of them in detail, but they have been nicely summarised in a series of recent reports [44-46]. One of the most powerful methods, particularly suitable for the preparation of water-soluble CNTs, is the 1,3-dipolar cycloaddition of azomethine ylides [47-49]. Figure 2 represents the molecular structure of a *f*-SWNT obtained from this type of cycloaddition reaction, with the organic moieties homogeneously distributed around the tube surface. By carefully choosing the reagents, it is possible to introduce amino groups around the CNT (for example, see the molecular structure of *f*-CNT 1 in Figure 3). This reaction works efficiently with both SWNTs and MWNTs. However, SWNTs show a loading of 0.3 – 0.5 mmol of functional groups/g of material, whereas MWNTs carry about 0.5 – 0.9 mmol/g. The higher loading for MWNTs could be attributed to the fact that they are isolated entities and present a larger surface area available during the addition reaction in comparison with the SWNTs, which instead form bundles that are difficult to disaggregate.

Many different functional groups can be covalently linked to CNTs. An example of a versatile intermediate that can be very easily subsequently derivatised is compound 1 in Figure 3. *f*-CNT 1 is interesting because it is highly soluble in aqueous solutions. It is of a cationic character, which suggests the possibility of utilising it as a new system for the development of innovative vectors for drug or vaccine delivery. To prove this possibility, a series of amino acids, fluorescent probes and bioactive peptides have been covalently linked to CNTs through the amino groups in 1 (derivatives 2 – 6, Figure 3). In particular, the approach of having the peptides linked to CNTs via a covalent bond was driven principally by the perspective of using CNTs for vaccine delivery (*vide infra*). In addition, *f*-CNT 1 can be considered a useful component in the formation of supramolecular complexes with nucleic acids and plasmid DNA, stabilised by positive–negative electrostatic interactions, for gene delivery.

3. Endohedral carbon nanotubes

Before describing the applications of *f*-CNT for drug, vaccine and gene delivery by exploiting the conjugation and complexation with various molecules on their external walls, an alternative approach to delivery using the internal cavity of the CNT will be addressed. Endohedral CNTs are non-covalent complexes in which the internal hole of the CNT is filled with various molecules. These molecules can be simply encapsulated or they can migrate along the tube.

3.1 Molecular encapsulation inside carbon nanotubes

The size and geometry of the CNT provide unique opportunities to create systems in which different molecules can be encapsulated into the nanotube hole [50-52]. Luzzi and coworkers have discovered that fullerene can be hosted, for example, into CNTs [53]. Nanotube doping is a very promising approach towards the conception of new devices for molecular electronics. Besides fullerenes [54], other classes of molecules have been recently integrated into CNTs, including polyaromatic and polyheteroaromatic condensed systems [55]. Host–guest complexes between CNTs and organic molecules have been limited to applications in nanoelectronics, but such supramolecular systems certainly hold a lot of potential if we imagine entrapment of bioactive molecules and their flow through the tubes.

3.2 Molecular migration through carbon nanotubes

The formation of endohedral CNTs has opened the possibility of creating interesting dynamic systems in which molecules move throughout the tubes. We can imagine that eventually the migrating molecule can travel from one end of the tube to the other. It has been demonstrated by molecular dynamics simulation that water can spontaneously fill and traverse the non-polar CNT cavity, suggesting the potential for their use as molecular channels [56,57]. In a similar approach, molecular simulations have shown that small oligonucleotides and polyethylene chains can also spontaneously penetrate into CNTs [58,59]. Based on these theoretical studies, several applications could be envisaged, including: the fabrication of molecular sensors; the electronic DNA sequencing; and the nanofabrication of gene delivery systems. To support this idea, the migration of DNA through the channel of a MWNT has been directly observed using fluorescence microscopy [60]. Recently, ionic flow and electro-osmotic phenomena across CNTs were simulated with the goal of mimicking the artificial

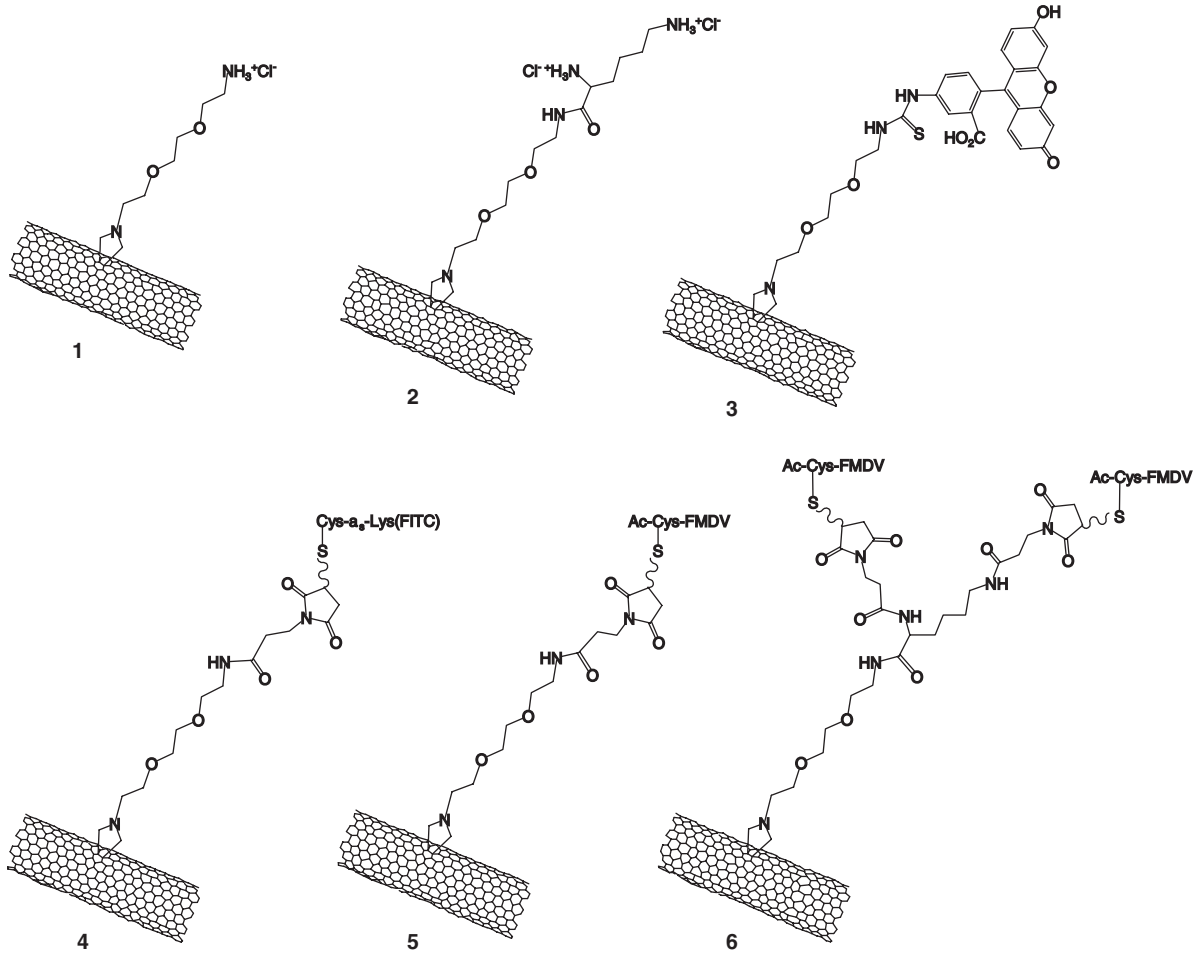

Figure 3. Structural drawings of functionalised carbon nanotubes.

protein channels found in cell membranes using CNTs [61,62]. Although very promising, these results need further confirmation and, above all, implementation into biological systems. It has been reported that CNTs can also be used as channel blockers [63]. This is somehow in contrast with the possible potential of CNTs to allow communication between the cytoplasm and the extracellular environment. In addition, the technology that exploits the cavity of CNTs for delivery bears the limitation of the extremely low solubility of the system for acceptable integration into physiological conditions.

4. Functionalised carbon nanotubes for drug delivery

The investigation of the potential applications of soluble *f*-CNTs as novel drug delivery systems has been suggested by their macromolecular and cationic nature. A fundamental prerequisite for a vector to deliver any type of therapeutic is its capacity to penetrate efficiently into the cell. *f*-CNTs, modified with either fluorescein isothiocyanate (FITC) or a fluorescent peptide (for their molecular structures see conjugates 3 and 4 in Figure 3), are able to cross the cell membrane and to localise into different cell compartments [64]. The capacity of internalisation of conjugates 3 and 4 was assessed by incubation with different types of cells, including human and murine fibroblasts, keratinocytes and HeLa cells, and by analysis with fluorescence microscopy. Whereas *f*-CNT–FITC was found to mainly distribute in the cytoplasm, the peptide–CNT was localised into the nucleus. Intracellular localisation of ammonium-functionalised MWNTs was detected using transmission electron microscopy (TEM). This alternative technique is particularly suitable when the fluorescent probe is absent [65]. Although the elucidation of the mechanism of entry requires further investigation, endocytosis was excluded because inhibitors of endosome-mediated translocation, and the decrease of the incubation temperature, did not impede

the cellular uptake of the *f*-CNTs. TEM images showed that the tubes cross the cell membrane as nanoneedles without perturbing or disrupting the membrane. This mechanism is supported by a dynamics simulation study describing the insertion of amphiphilic nanotubes into lipid bilayers via a passive mechanism [66].

Parallel to the study of the capacity of *f*-CNTs to enter into the cells, a preliminary *in vitro* analysis of *f*-CNT cytotoxicity was reported [64]. Whereas the insolubility and the presence of the metal catalysts were proven to be responsible for cell mortality and tissue damage of nonfunctionalised CNTs derived directly from the manufacturing [41-43], *f*-CNTs are instead highly soluble and display low cell toxicity [64,65]. The cell cultures were treated with increasing doses of *f*-SWNTs and *f*-MWNTs, and for long incubation times. Only very high doses of nanotubes (5 – 10 mg/ml) cause a mortality of 50 – 90% of the cells [65]. The capacity of *f*-CNTs to cross the cell membrane with reduced toxicity has been also demonstrated by Wender and Dai [67]. CNTs were made soluble by treatment with strong acids (oxidation process [68]) and sonication. In this case, carboxylic functions were generated at the side walls and at the ends of CNTs. These groups were derivatised with either FITC or biotin [67]. The CNTs with the biotin were subsequently conjugated to a fluorescent streptavidin. The strong streptavidin–CNT complex entered into the cell via an endocytosis mechanism. Although this behaviour is in contrast with the internalisation process found for conjugates 3 and 4, endocytosis is likely to be controlled by streptavidin. However, with the available data it is difficult to propose a general mechanism of CNT cellular uptake. These results represent the first demonstration of the potential use of *f*-CNTs as carriers for drug delivery, but they need to be improved and complemented with studies on *f*-CNT metabolism, biodistribution and clearance from the body. Depending on the different mechanisms of internalisation and the different distribution inside the cell governed by the functional groups (FITC, peptides, proteins) linked to the external walls of the CNTs, it could be possible to selectively target the various cellular compartments.

5. Functionalised carbon nanotubes for delivery of antigens

In the field of vaccination, many efforts are being devoted to the development of novel systems for the delivery of protective antigens. This is mainly due to the fact that immunisation with synthetic vaccines, based, for example, on peptides, elicits weak immune responses in comparison with natural pathogens. Within the class of non-replicating vectors, *f*-CNTs could be considered as an alternative carrier system for peptide antigens. Indeed, *f*-CNTs are carbon polymers (they can be imagined as a repetition of condensed benzene rings) with a high loading capacity for cargo molecules. In this context, synthetic peptides, considered as potential vaccine candidates, could be conjugated to CNTs to study the immunological properties of these systems. For this purpose, a model antigen representing a B cell epitope from the foot-and-mouth disease virus (FMDV) was coupled to CNTs [49]. The peptide was linked to the *f*-CNT 1, either as a mono-conjugate (via a maleimido spacer) or as a bis-conjugate using a lysine (for their molecular structures see conjugates 5 and 6 in Figure 3) [49,69]. Peptide–CNTs 5 and 6 were recognised by the monoclonal and polyclonal antibodies as well as the free FMDV peptide, suggesting that the folding of peptide attached onto the nanotubes was similar to the conformation the peptide adopted free in solution [49,69]. Therefore, the peptide was presented by the nanotubes in the appropriate conformation for the spatial interaction with the antibodies. Very important was the fact that *f*-CNTs devoid of the peptide moiety did not interact with the antibodies. Concerning the immunogenic properties, *f*-CNTs 5 and 6 elicited significantly higher anti-FMDV peptide antibody responses than the free peptide and the mixture of the free peptide with *f*-CNT 1 [69]. In addition, no antibodies against *f*-CNT 1 (devoid of peptide moiety) were detected. This suggests that CNTs do not possess an intrinsic immunogenicity. The lack of immune response to CNTs is important in view of the epitopic suppression phenomenon often observed after several administrations of peptide antigens coupled to carrier proteins. Studies by Erlanger and co-workers have demonstrated that antibodies induced against the fullerenes [70] can recognise CNTs [71]. However, the fullerenes were immunogenic only after being coupled to a carrier protein [70], whereas in the case of the antigens onto the *f*-CNTs, a co-immunisation protocol has been used [69]. The elicited antipeptide antibodies also had virus-neutralising capacity. *f*-CNT 5 induced significantly higher neutralising responses than those induced by *f*-CNT 6, although the latter generated higher antibody titres. These antibodies probably lacked the correct specificity, as the two copies of the FMDV peptide bound to the CNTs might interact *in vivo* and adopt a conformation different from that displayed by the native epitope on the virus. These data are in agreement with previous observations showing the limitation of multiple presentation of epitopes to elicit antibodies cross-reactive with the native protein [72]. In view of these results, *f*-CNTs can be considered as an interesting and promising carrier system for the delivery of candidate vaccine antigens based, for example, on peptides and proteins.

6. Functionalised carbon nanotubes for delivery of genes

Gene therapy is one of the most promising approaches to treat different diseases, such as cancer or genetic disorders [73]. However, there are some limitations, mainly related to the pharmacokinetic characteristics of the oligonucleotides and plasmid DNA. These biopolymers are rapidly degraded by nucleases and display a poor bioavailability profile [74]. Many research efforts are focused towards the development of an effective delivery system for nucleic acids [75]. DNA delivery is

Table 1. Characteristics of the functionalised carbon nanotubes in comparison with micro- and nanoparticles.

	Functionalised carbon nanotubes	Micro/nanoparticles
Shape	Tubular form	Spherical form
Manufacturing	Easy fabrication and processing	Difficult large-scale production
Modification (i)	Functionalisation with different groups	Functionalisation with different groups
Modification (ii)	Molecules can be adsorbed or linked to the external walls. There is the potential to insert them into the tube cavity	Molecules can be attached or adsorbed at the surface or encapsulated
Modification (iii)	Good control of conjugation	Low loading capacity
Routes of administration	Potential administration via all routes	Administration via different routes (mucosal, systemic, transcutaneous)
Biocompatibility/biodegradability	Biocompatible/non-biodegradable	Biocompatible/biodegradable
Cell uptake	Good	Good
Cytotoxicity	Low toxicity (tested only *in vitro*)	Low toxicity
Immunogenicity	Absent	Absent
Adjuvant effect	Absent	Absent
Storage	Not tested	Instability of the active compounds into the spheres
Controlled release	Potential (possibility of tailor-made delivery according to the needs)	Critical to control drug release rates
Active delivery of molecules	Oligodeoxynucleotide, plasmid DNA	Oligodeoxynucleotide, plasmid DNA, antigens

currently based on the use of viral and non-viral vectors. Concerns have arisen for the former, as this type of vector can induce undesirable immune responses, inflammation and oncogenic effects. For these reasons, non-viral vectors have been highly explored, as they can guarantee a higher degree of safety. This family of vectors includes liposomes, cationic lipids, micro- and nanoparticles, and many others [76,77].

On the basis of the different classes of available gene carriers, *f*-CNTs have been fashioned into the development of a new delivery system for gene transfer. In fact, it is possible to exploit the macromolecular and cationic nature of the *f*-CNTs to form supramolecular complexes with plasmid DNA, eventually tested in gene transfer and gene expression experiments. Previous studies on the interaction of CNTs and DNA were focused to increase the solubility of nanotubes in aqueous solution and to reduce their polydispersity. For example, it has been demonstrated that SWNTs assemble with single-stranded DNA [34,35]. In addition, CNTs have been functionalised at their terminal parts with single-stranded DNA or peptide nucleic acid, and hybridised with the complementary DNA sequences to form supramolecular nanotube-based structures [78-81].

For exploring the potential of CNTs as gene transfer vectors, the capacity of the *f*-CNT 1 to condense the plasmid DNA has been initially elucidated [65]. *f*-CNT 1 was able to form a complex with pCMV-βgal, which expresses β-galactosidase, stabilised by ionic interactions. In the supramolecular assembly of the two components, CNTs were present in bundles of different diameters, and plasmid DNA was absorbed onto the CNT clusters by forming toroidal, globular or supercoiled structures [65]. Following the formation of the complex, gene transfer experiments have shown a clear effect of *f*-CNT 1 on the expression of β-galactosidase [65]. Levels of gene expression 5- to 10-times higher than that of DNA alone were obtained. Preliminary comparative gene expression data between *f*-CNT–DNA and lipid–DNA delivery systems showed that this first generation of *f*-CNTs is less effective for transfection *in vitro* than commercially available systems. Therefore, *f*-CNT technology for gene transfer requires further improvements. However, these promising data could represent the first step towards the exploitation of *f*-CNTs in gene therapy and genetic vaccination.

7. Expert opinion

The development of novel delivery systems for the successful administration of different classes of therapeutic molecules is comprised in the list of the top 10 biotechnologies for improving global health, particularly in developing countries [82]. Delivery based on *f*-CNTs is a new technology, which is still in its infancy for biomedical applications. However, on the basis of their interesting properties, which have recently been reported, *f*-CNTs hold a great potential for application in drug, vaccine and gene delivery. Indeed, *f*-CNTs are compatible with biological systems. In particular, their surface functionalisation enables non-covalent adsorption or covalent attachment of different molecules or antigens, which subse-

quently can be targeted to the desired cell population for immune recognition or a therapeutic effect.

At this stage, it is not possible to make a direct comparison with other existing delivery technologies that are available and have been exploited for many years. Table 1 summarises, for example, the characteristics of *f*-CNTs in comparison with micro- and nanoparticles.

From Table 1 it is evident that *f*-CNT technology for drug delivery requires further investigation for validation, mainly concerning certain important aspects such as the toxicity *in vivo*. Although cytotoxicity issues have so far only been tackled *in vitro*, there are potentially practical benefits in developing novel vectors based on *f*-CNTs for medical applications. For example, *f*-CNTs can enhance the bioavailability of the attached antigens and can protect complexed oligonucleotides from degradation during gene transfer. In addition, besides the adsorption of plasmid DNAs the formation of complexes with oligodeoxynucleotide CpG motifs, which are being explored as new adjuvant candidates for their promising immunostimulatory properties, can be envisaged. Cationic *f*-CNTs could also have potential applications for mucosal immunisation due to the possibility of a good interaction with the negatively-charged mucosal epithelium. This has been supported by using, for example, chitosan formulations for nasal delivery of vaccines [83]. Finally, as it is difficult to imaginge and conceive a universal delivery system, *f*-CNTs can complement existing technologies. New investigations and the constant progress on the physicochemical and biological properties of *f*-CNTs will facilitate and reinforce the application of this type of material as an innovative and effective delivery system.

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