Carbon Nanotubes: On the Road to Deliver

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Abstract: Over the last few years, considerable advances have been made in the field of nanotechnology. The advent of carbon nanotube functionalization has paved the way for their potential application as a delivery system of diverse molecules such as peptides, proteins, plasmid DNA, and synthetic oligodeoxynucleotides. This opens new therapeutic and preventive opportunities to combat diseases. The scope of this review is to summarize our recent work in this rapidly growing field.

Keywords: Carbon nanotubes, delivery system, vaccine, plasmid DNA, ODN CpG.

1. INTRODUCTION

The immune system has evolved to respond to pathogens bearing molecules organized to ensure protection of their genome, and eventually facilitate its delivery into the cells for replication. Upon entry to the host, these molecules trigger immune responses aiming to destroy the pathogen and confer protection to the individual. Until now, this simple principle formed the basis for the development of vaccines comprising the whole microorganism either killed or attenuated. For safety reasons, a lot of effort has been made to replace the whole microorganism with subunit vaccines, selecting only those antigens or parts of them (epitopes), that elicit a protective immune response. It is therefore not surprising why immunization with subunit vaccines or peptides that have much lower ordered structure often results in the induction of immune responses inferior to those elicited after natural infection. However, recent biotechnological advances have provided us with a new generation of delivery systems based on replicating or non-replicating vectors aiming to enhance their immunogenicity.

Replicating vectors are based on recombinant bacteria (i.e. *Salmonella Dublin*, *Shigella flexneri*, *Listeria monocytogenes*) or viruses (i.e., vaccinia and related avipox viruses, adenovirus strains) expressing epitopes or protein antigens [1-4]. Such vectored vaccines being live, are likely to share with the pathogen, the same antigen processing and presentation pathways therefore, they have the potential to elicit the desired type of protective immune responses. Live attenuated vectors induce long lasting systemic and/or mucosal immunity and can be produced in large quantities. Despite their advantages, this type of delivery systems pose several risks due to their invasive capacity. The viral genome could become integrated into the host chromosomes with the potential risk of insertional mutagenesis through the activation of an endogenous proto-oncogene or deactivation of tumor suppressor genes in the host. Moreover, reversion to virulence is always a major concern for attenuated pathogens and the effect of multiple uses on vector efficacy has to be determined. The use of non-replicating vector systems could eliminate some of these concerns. An ideal non-replicating vector should protect molecules from degradation, ensure efficient uptake by the antigen-presenting cells, and be biocompatible and biodegradable, and not immunogenic. Particles like liposomes [5], immune stimulating complexes [5], virosomes [6], biodegradable microparticles [7] and chitosan-based nanoparticles [8] have been extensively tested for vaccine delivery with various degrees of success. This was dependent on the nature of antigen, the formulation, and the route of administration.

The development of new delivery systems for the successful and effective administration of vaccines and immunotherapeutics still remains a great challenge ahead. This view has recently been reinforced by listing delivery among the top ten biotechnologies required for improving global health [9]. In this review, we summarize our recent data on the potential use of carbon nanotubes (CNT) for the delivery of peptide antigens, plasmid DNA and synthetic oligodeoxynucleotides (ODN) containing CpG motifs (ODN CpGs).

2. CARBON NANOTUBES

CNT belong to the family of fullerenes, the third allotropic form of carbon along with diamond and graphite [10]. They are comprised exclusively of carbon atoms arranged in a condensed polyaromatic surface rolled-up in a tubular structure with their ends closed. CNT exists in two main forms: i) single-walled carbon nanotubes (SWNT) with a sole cylindrical graphene sheet, and ii) multi-walled carbon nanotubes (MWNT) comprising of several concentric graphene sheets, spaced by a distance of about 0.34 nm (Fig. **1**) [11]. CNT were discovered in the late 1950s [12]. However, it was only in the last decade of the 20th century, thanks to

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the Japanese researcher Sumio Iijima, that CNT became a subject of intense research interest [13].

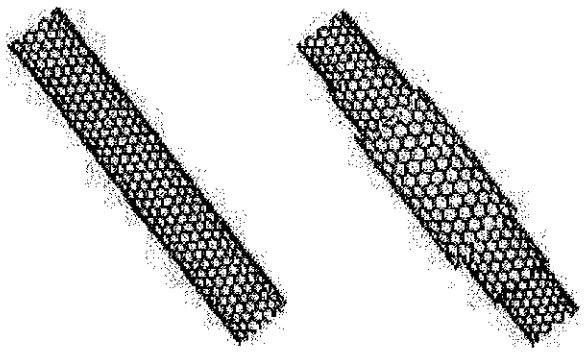

Fig. (1). Molecular structures of a fragment of SWNT (left) and MWNT (right).

Initially, CNT were considered mainly for applications in the field of material science because of their mechanic, electronic, optic and magnetic properties [14]. Soon after, CNT were examined for their potential applications in the fields of biological and biomedical sciences [15-17]. However, it was realized that the intrinsic property of CNT of poor solubility in all types of solvents, hindered their integration into biological systems. The development of methodologies that enabled their covalent and non-covalent functionalization has finally solved this problem. In fact, it is now possible to form supramolecular complexes between CNT and peptides, proteins, oligosaccharides, oligonucleotides and other types of polymers, which are able to wrap around the tubes and therefore, increase their solubility (Table **1**) [18-33]. An alternative powerful approach is to covalently link different functional groups around the sidewalls and the end parts of the nanotubes (Table **1**) [34-63]. Following different synthetic procedures, CNT can be modified by generating carboxylic or amine functions that can subsequently be derivatized with bioactive molecules (Table **1**). As a result, the use of CNT and particularly functionalized CNT (*f*-CNT) as vectors for the development of a new delivery system could be envisaged.

3. TOXICITY OF *F*-CNT

Recently, several concerns about the toxicity of CNT have been raised [64-67]. This is true for the non *f*-CNT. Once they form complexes with bioactive molecules and after their internalization and release of the cargo molecule into the cells, the free CNT aggregate and precipitate. This toxic effect on cells and organs is partly due to the insolubility of non *f*-CNT and the presence of catalyst particles that are necessary for their production. Therefore, this property poses serious limitation for their use as vectors. However, when molecules are covalently linked to *f*-CNT, their toxicity is significantly reduced and they are safe and suitable for biological applications [68, 69]. Fig. **2A**, shows a representative experiment where various concentrations of ammonium-functionalized multi-walled carbon nanotubes (MWNT-NH_3^+·Cl^-) had no significant effect on the ^{3}[H]-thymidine uptake by splenic lymphocytes. In contrast, non-*f*-MWNT were toxic (Fig. **2B**). Moreover, MWNT-NH_3^+·Cl^- did not affect the concentration of secreted IL-6 (Fig. **3A**) and IFN-_ (Fig. **3B**), measured in culture supernatants of mitogen (ConA)-stimulated splenocytes. No proliferation or IL-6 and IFN-_ secretion was detected in cultures of non-activated mouse splenocytes in the presence of various concentrations of MWNT-NH_3^+·Cl^- (Fig. **3A** & **3B**), suggesting that they are not mitogenic. In a similar set of experiments, we also observed no toxicity with ammonium-functionalized single–walled carbon nanotubes (SWNT-NH_3^+·Cl^-) and lysine-functionalized single-walled carbon nanotubes (SWNT-Lys-NH_3^+·Cl^-) [70]. These preliminary data indicate that *f*-

Table 1. Main Strategies for Non Covalent and Covalent Functionalization of CNT

Non covalent CNT functionalization	Ref.	Covalent CNT functionalization	Ref.
Poly(vinylpyrrolidone)	[18]	Oxidative treatment	[39, 40]
Poly(*m*-phenylenevinylene)	[19]	Fluorination/Amination	[41,42]
Surfactants	[20-22]	Alkyl halides	[43, 44]
Poly(styrenesulfonate)	[18]	Radical addition/polymerization	[45-48]
Polysaccharides	[23, 24]	Polymer grafting	[49, 50]
Cyclodextrins	[25, 26]	Reduction of diazonium salts	[51-53]
Soy oil	[27]	Solvent-free amination	[54, 55]
Oligonucleotides	[28, 29]	1,3-Dipolar cycloaddition	[56-58]
Peptides	[30, 31]	Electrophilic addition	[59]
Gum Arabic	[32]	Carbene addition	[45, 60, 61]
Nafion	[33]	Nitrene addition	[45, 62]
		Bingel reaction	[63]

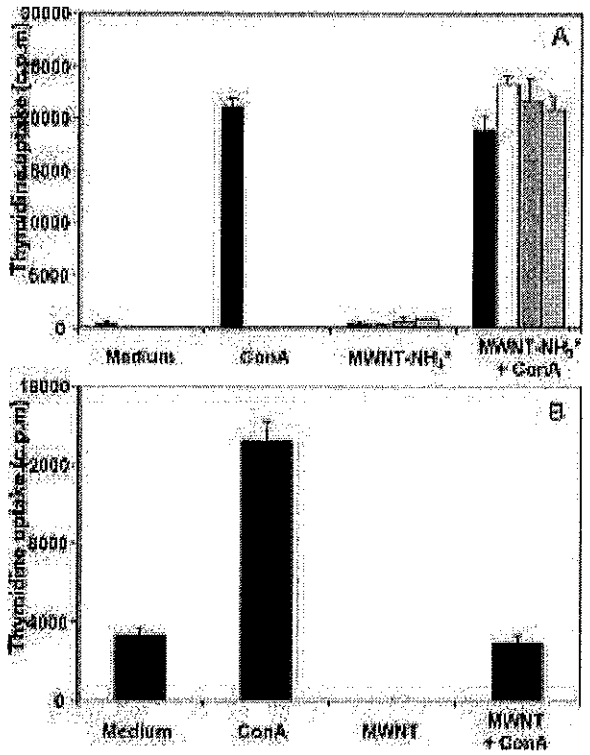

Fig. (2). Thymidine uptake by ConA-activated or non-activated mouse splenocytes cultured with various concentrations [5 μg (black), 0.5 μg (white), 0.05 μg (dark grey) and 0.005 μg (light grey)] of MWNT-NH_3^+·Cl^- (A) and one concentration (5 μg) of non functionalized MWNT (B).

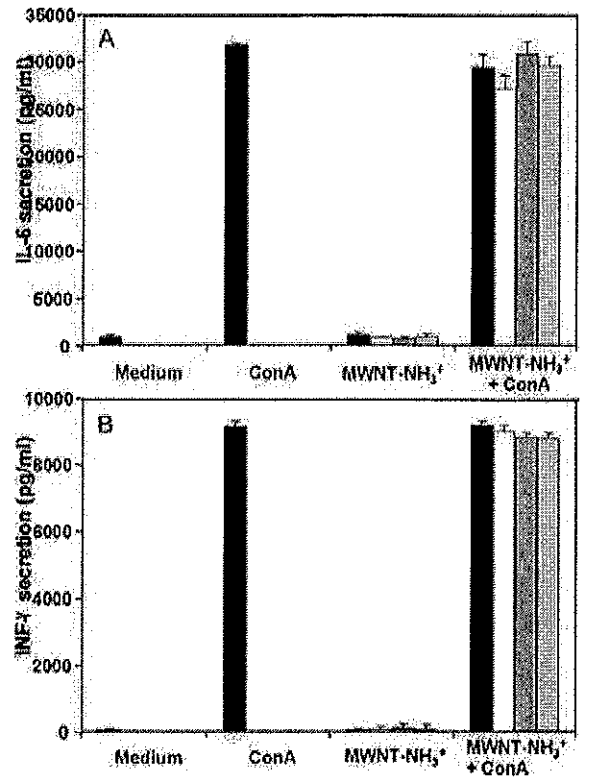

Fig. (3). IL-6 (A) and IFN-γ (B) secretion by ConA-activated or non-activated mouse splenocytes cultured with various concentrations [5 μg (black), 0.5 μg (white), 0.05 μg (dark grey) and 0.005 μg (light grey)] of MWNT-NH_3^+·Cl^-.

CNT could be safe for potential biomedical applications. Furthermore, they confirm results from *in vitro* studies on *f*-CNT cytotoxicity [68, 69]. Water-soluble *f*-CNT displayed low cellular toxicity after treating cell cultures with increasing concentrations of ammonium-functionalized SWNT for long incubation periods. Only high concentrations of *f*-CNT (5-10 mg/ml) caused cell mortality that reached up to 50-90% [69].

4. DELIVERY OF DNA AND ODN CPGS WITH *F*-CNT

4.1 Interaction of Cationic CNT with ODN CpG and Plasmid DNA

The elucidation of the structural and functional relationship between biomolecules is a challenging issue in modern biology. Surface plasmon resonance technology (SPR) that allows the study of interaction between two molecules in real time, has given a new insight in better understanding how two molecules bind. In our studies, we used Biacore technology to analyze the interaction of synthetic oligodeoxynucleotides (ODN) containing immunostimulatory CpG motifs (ODN CpG) or plasmid DNA with cationic CNT [70]. CpG motifs are unmethylated sequences that derive from bacterial DNA and have immunostimulatory properties [71]. Given these properties, synthetic ODN CpGs have been considered as candidate adjuvants for vaccines or immunomodulators for therapeutic applications against tumors, allergy or to combat bioterrorist threats [72-75].

In the first series of experiments, we selected three types of *f*-CNT; the SWNT-NH_3^+·Cl^-, the MWNT-NH_3^+·Cl^- and the SWNT-Lys-NH_3^+·Cl^-. Each *f*-CNT was attached onto the sensor chip by forming a stable amide bond between the amino groups on the tubes and the carboxylic functions on the chip's carboxylated dextran matrix, activated in turn with carbodiimide and N-hydroxysuccinimide. Following attachment, the increase in mass due to the interaction of *f*-CNT with the ODN CpG 1668 present in the fluid phase was measured using the Biacore 3000 system [70]. Although there were no significant differences between the affinity of binding between the two SWNT preparations and the ODN CpG 1668, the ODN CpG bound to the MWNT-NH_3^+·Cl^- formulation with much lower affinity (Fig. **4**). This difference is probably due to the relative dimension and charge distribution of the two types of *f*-CNT. Both SWNT-Lys-NH_3^+·Cl^- and MWNT-NH_3^+·Cl^- display the same amount of positive charges. However, there must be an avidity effect, resulting from a double electrostatic interaction per ODN CpG molecule after their binding to SWNT-Lys-NH_3^+·Cl^-, where the two ammonium groups on each lysine residue are in close proximity in respect to the single ammonium on the MWNT-NH_3^+·Cl^-.

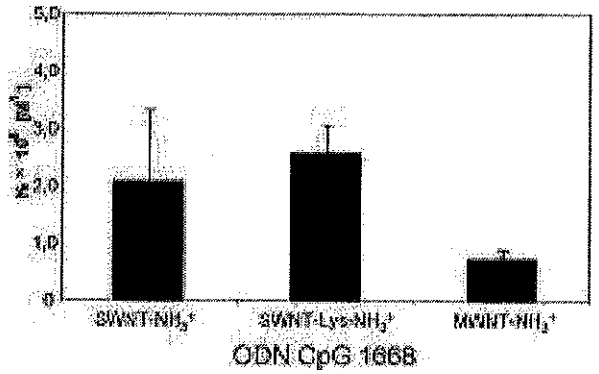

Fig. (4). Comparison of the equilibrium association constants for the formation of complexes between the different *f*-CNT and the ODN CpG 1668.

Biacore experiments using plasmid DNA in the fluid phase, established that the plasmid could bind with much higher affinity to SWNT-Lys-NH_3^+·Cl^- immobilized onto the sensor chip as compared to the MWNT-NH_3^+·Cl^- preparation [76]. Overall, these results highlighted the fact that the positive charge of *f*-CNT supports their interaction with the negatively charged ODN CpG or plasmid DNA, and paved the way to examine the delivery potential of *f*-CNT.

4.2 Enhanced Transfection with Plasmid DNA Coated onto *f*-CNT

The early demonstration by Tang *et al.*, that a simple injection of naked DNA induces an antigen-specific immune response paved the way for the development of a new immunization strategy commonly termed as genetic or DNA vaccination [77]. In comparison with transfection strategies based on delivering genes *in vivo* with viral vectors, the use of naked DNA offers several advantages including, safety (i.e., no infection, mutagenesis or unwanted immune responses to viral proteins), capability for simultaneous administration of multiple genes encoding different molecules (i.e., antigens, immunomodulating cytokines, costimulatory molecules), and feasibility to generate large scale production of plasmid DNA. Although direct injection of plasmid DNA has been shown to lead to gene expression, the overall levels of encoded protein were much lower compared to its production with viral or liposomal vectors [78]. Since cationic carriers are widely accepted as effective delivery systems because of their capacity to condense DNA and interact with cells [79], positively charged *f*-CNT were used to deliver DNA to cells. In our studies, we observed that *f*-CNT were capable to condense plasmid DNA (Fig. 5) [69]. Electrostatic interactions between the DNA and *f*-CNT could maintain the complex together, to warrant intracellular translocation. It was recently shown in our laboratories that interaction between *f*-CNT and plasmid DNA can lead to delivery and transfection of mammalian cells (Fig. 6) [69]. This finding paves the way for the use of *f*-CNT as a novel system for the delivery of genes with direct applications on gene therapy and genetic vaccination.

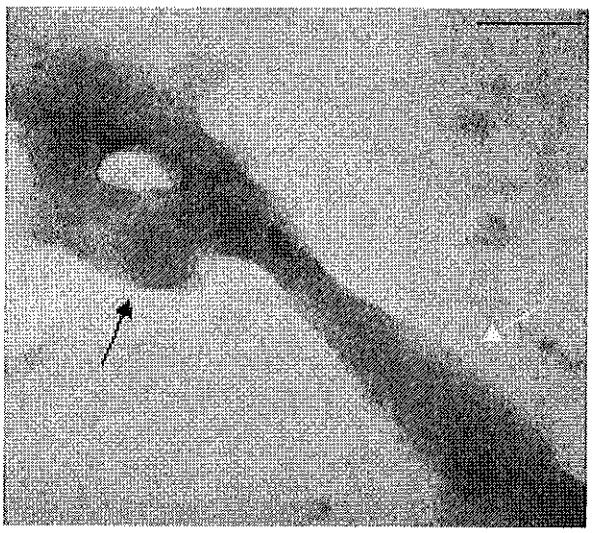

Fig. (5). Complexation between SWNT-NH_3^+·Cl^- and plasmid DNA as imaged by negative stain electron microscopy. The DNA (black arrow) is condensed into a bundle of nanotubes (white arrow) by electrostatic interactions. Scale bar is 100 nm.

4.3 *f*-CNT Improve the Immunostimulatory Properties of ODN CpGs

The triggering of the Toll-like receptor 9 by the ODN CpG is a critical event for the activation of innate immune system [71]. This requires the entry of ODN CpG into the cell for recognition by its receptor, which is expressed in endosomal compartments [80]. However, despite the potent immunostimulatory properties of ODN CpGs, their biological activities are short-lived. This is because their intracellular delivery faces the challenge of low uptake by the cells, due to the negative charge of cell membranes. In order to increase their biological properties, we complexed them with *f*-CNT and tested their immunostimulatory properties *in vitro*. In our studies we selected SWNT, since they were shown to bind to ODN CpG 1668 with higher affinity than the MWNT [70]. After incubating the SWNT-Lys-NH_3^+·Cl^- at various ratios with a minimal immunostimulatory dose of ODN CpG 1668, the complexes were added to a culture of naïve mouse splenocytes. SWNT-Lys-NH_3^+·Cl^- at 18:1 and 9:1 ratios over the ODN CpG 1668 was shown to increase its immunostimulatory properties by 58% and 45%, respectively. There was no significant effect on immunostimulation when lower ratios of *f*-CNT/ODN CpG were tested [70]. Although the mechanism of this immunopotentiating effect is not quite clear, it could be argued that the high excess of SWNT-Lys-NH_3^+·Cl^- over ODN CpG 1668 has neutralized its negative charge. As a consequence, the repulsion by the negatively charged cell membrane was presumably reduced and therefore, the cellular uptake of ODN CpG 1668 was increased. These preliminary findings are very encouraging and future experiments *in vivo* using animal models of disease or vaccination protocols to test the therapeutic or adjuvant properties of such complexes would hopefully pave the way to use *f*-CNT in clinical settings.

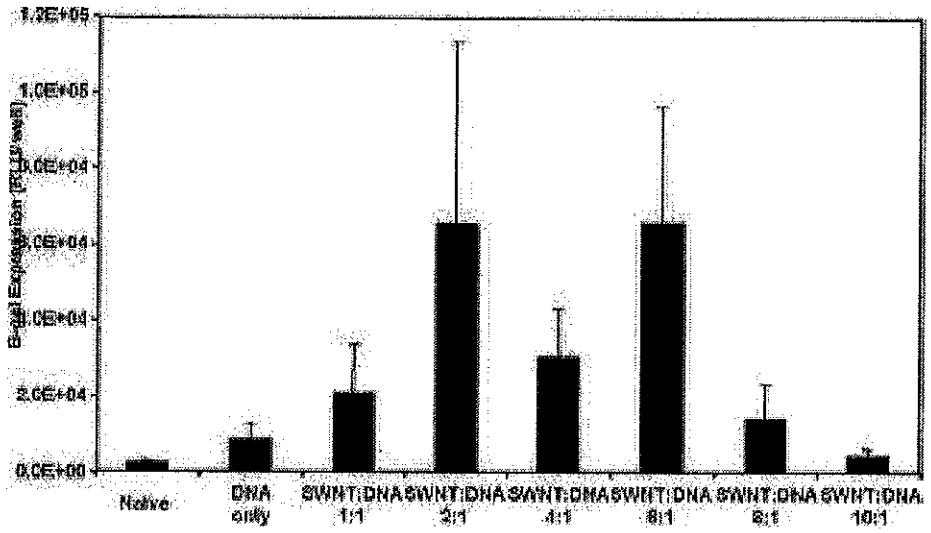

Fig. (6). Gene expression in CHO cells following transfection with plasmid DNA encoding the reporter gene for ß-gal complexed with SWNT-NH_3^+·Cl^-. All data shown refer to gene expression following a 3 hour incubation between the SWNT-NH_3^+·Cl^-:DNA complexes and cells.

5. PRESENTATION OF PEPTIDE ANTIGENS WITH *F*-CNT

The antibody response to a protein antigen is predominantly focused to restricted parts of the molecule termed B cell epitopes. These epitopes are mainly located on the surface of the molecule and thus, the native structure of the antigen is critical for this interaction to occur [81]. The demonstration that peptides can induce antibodies of predetermined specificity, has prompted to examine their potential as candidate vaccines [82]. Immunization with unconstrained peptides normally induces a diverse set of antibody specificities that bind several different conformations of the peptide. However, a subset of these antibodies can cross-react with the native protein, presumably because these peptides adopt a conformation similar to the native epitope. Therefore, for a synthetic peptide vaccine to elicit a protective antibody response, it is important the B cell epitope to be presented to the immune system at a conformation mimicking the native structure.

In our studies, we tested the ability of *f*-CNT to present a covalently linked synthetic peptide representing a neutralizing and protective B cell epitope from the foot-and-mouth disease virus (FMDV) corresponding to the 141-159 region of the VP1 viral envelope protein [83]. To this end, we used SWNT-NH_3^+·Cl^- and SWNT-Lys-NH_3^+·Cl^- modified with a maleimido group. This functionality allowed linking the FMDV peptide bearing an additional cysteine at the N-terminus necessary for a selective chemical ligation [84]. To elucidate the structural and functional relationship between CNT attached peptide and peptide-specific polyclonal and monoclonal antibodies, we used a solid phase immunoabsorbent assay (ELISA) and SPR technology. Both methods demonstrated that the SWNT-linked peptide retained intact its antigenicity, since it was recognized equally well as the free peptide by anti-peptide monoclonal and polyclonal antibodies [84]. This suggests that the structural features adopted by the peptide epitope when attached to SWNT, achieved the required complementarity between the paratope (the binding site of antibodies) and the B-cell epitope. Since the FMDV peptide was not immunogenic (capable of eliciting an immune response) in BALB/c mice (requires T cell help) [85], we employed an immunization protocol that has previously been shown to overcome the requirement of coupling non-immunogenic peptides to carrier proteins or to T helper epitopes [86]. To this end, the SWNT-linked FMDV peptide was co-immunized with a protein antigen that can provide T-cell help, like the ovalbumin (OVA) in a Freund's emulsion. After two injections, strong anti-peptide antibody responses were induced that had significantly higher virus neutralizing capacity than the antibodies elicited after co-immunization of the free peptide with OVA [87]. This finding highlights the potential of *f*-CNT to act as a delivery system capable of presenting critical epitopes at an appropriate conformation to elicit antibodies with the right specificity. Moreover, no antibody responses were induced against the *f*-CNT, which could potentially hamper the successful outcome of the immunization procedure, particularly when several administrations are required [88].

6. CONCLUSIONS

Recent biotechnological advances promise to contribute to the prevention of disease and the promotion of health. Among the emerging novel technologies with potential to improve the delivery of vaccines and many immunotherapeutics is nanotechnology based on CNT. Our preliminary results suggest that we can harness the interesting properties of these molecules for biomedical application and particularly to deliver intracellular genes and oligodeoxynucleotides and present protective epitopes at an appropriate conformation [89]. It is true that much more work is needed to prove the validity of these early findings *in vivo,* but the challenge has already been set by us and other groups working in this field, to make CNT another member of the arsenal, which

will contribute in improving conventional public health strategies such as vaccination and therapy [90].

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