

REVIEW

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Carbon nanotubes as a novel tool for vaccination against infectious diseases and cancer

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Abstract

Due to their unusual properties, carbon nanotubes have been extensively employed in electronics, nanotechnology and optics, amongst other. More recently, they have also been used as vehicles for drug and antigen delivery, the latter being a novel immunization strategy against infectious diseases and cancer. Here we discuss the potential of carbon nanotubes as an antigen delivery tool and suggest further directions in the field of vaccination.

Keywords: Vaccine, Carbon nanotubes, Functionalization, Infectious diseases, Cancer

Introduction

Carbon nanotubes (CNT) are probably the most famous members of the fullerenes family. Fullerenes comprise any molecule made entirely of carbon atoms, shaped as a sphere, an ellipsoid, or a tube [1]. In 1991, Iijima and colleagues published in *Nature* their description of “helical microtubules of graphitic carbon” [2]. CNT are generally distinguished between single walled carbon nanotubes (SWNT) and multiwall carbon nanotubes (MWNT). The former can be conceptualized as a seamless cylinder obtained by rolling up a single sheet of graphite generally referred to as a graphene layer, i.e., a plane of carbon atoms arranged in a hexagonal lattice. MWNT can be thought as the coaxial assembly of different SWNT of different diameters, one contained within each other. The characteristic aspect ratio of CNT is in the order of millions to one, as their length can span hundreds of microns whereas their diameter is only few nanometers across, and down to less than 0.8 nm for the smaller SWNT. Because of their unique geometry, CNT are often referred to as “one dimensional” [3] and even “zero dimensional” [4] objects.

Their nanometer lateral dimensions are the basis of several of the desirable properties of CNT. However, they also represent a potential limitation, contributing to the inherent difficulties of working with CNT. In fact,

pristine nanotubes are virtually insoluble in most solvents in ordinary conditions [5], which limits their large scale processability. Consequently, a large amount of research has been devoted to develop processes that could render CNT soluble, from functionalization of their hydrophobic sidewalls with soluble molecules [6], to wrapping individual CNTs with polymers [7] or DNA [8] and to reduce their length so their dispersibility can be increased [4,9]. Since their popularization in the early '90s, CNT have sparked a great deal of excitement in the scientific community and beyond, due to their unique properties, with potential to revolutionize the fields of material science, electronics, energy collection and storage, medicine and many more. CNT have unmatched tensile strength [10], which makes them the strongest material yet discovered, and great thermal conductivity along their axis [11]. In biomedical applications, CNT are mainly investigated either to enhance molecular biosensing [12], due to their optoelectronic properties [13-15], or as drug delivery agents [16]. Since CNT are readily internalized by cells [17], they are ideal vehicles for delivery of therapeutics [18] or diagnostics [19]. CNT can bind macromolecules such as proteins and oligosaccharides, which suggest they would have potential applications as carriers for delivery of active molecules, like drugs or antigens [20-22].

The functionalization of CNT is paramount, first to guarantee the solubility of CNT in aqueous solutions, crucial in a biological context and second, to ensure that the desired molecular agent, either a drug or a marker, is bound to the nanotube. As mentioned above, CNT are

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poorly soluble in water, and this has initially limited their use as a delivery agent for medicinal purposes [23]. Some approaches were then developed to improve their solubility. The most used is covalent functionalization, in which the desired active molecules bind to cationic functional groups, the latter bound either on the surface or within the walls of CNT. Examples of functional groups are polyethylene glycol (PEG) or ammonium-terminated triethylene glycol, which is a reactive intermediate that allows the synthesis of several functionalized CNT mixtures. Biomolecules such as oligonucleotides and proteins were also used to functionalize CNT. The functional groups are bound to the active molecules, increasing the solubility of CNT [24]. Furthermore, sugar moieties like starch and oligomers like polyvinylpyrrolidone wrap around CNT and transport them to the aqueous phase [7,22]. Both nanotube-mediated oligonucleotide transport inside living cells [25] as well as plasmid DNA gene delivery [26] have shown promising results, which might open the way to the use of CNT as non-viral delivery vectors. The use of CNT carriers could in fact overcome some of the limitations of other non-viral vectors available today, namely the poor pharmacokinetic profiles of the administered oligonucleotide and plasmid DNA conjugates, and the low levels of gene expression obtained [27].

Carbon nanotubes induce specific and protective Immune responses

An effective vaccine must induce a potent immune response, either at cellular level, stimulating cytotoxic T cells which target and destroy infected cells or at humoral level, through stimulation of the production of neutralizing antibodies which promote opsonization and consequent pathogen clearance. Vaccines against some pathogens, like HIV or Malaria, due to their complexity, would require both responses to be fully efficient in preventing infection and eliminating circulating pathogens. Innate immune responses are also important in vaccination, since they play a crucial role in antigen presentation and immune cells recruitment to infection sites [28].

Several cell types can uptake CNT, including cells of the immune system, such as macrophages, monocytes, natural killer (NK), dendritic cells, T and B cells [17,29-34]. In most cases, CNT did not impair functionality of these cells and it was observed that functionalized CNT are less toxic than pristine CNT, inducing therefore a lesser cytotoxic response [17,35]. CNT were shown to activate cells from the innate immune system, such as monocytes [29,31,34], macrophages [31,32,34] and dendritic cells [30]. Microarray profiling of a monocytic cell line, THP-1, showed that CNT, both functionalized and non-functionalized, activate several genes involved in monocyte response to infection or vaccination, such as nuclear factor kappa-light-chain-enhancer of activated B cells

(NF- κ B), interleukin-1 β (IL-1 β), IL-6, tumor necrosis factor- α (TNF- α), among others [29,31]. However, the non-functionalized CNT also increased the expression of genes related to oxidative stress and apoptosis [31]. The functionalized CNT were oxidized and further modified to incorporate ammonium; both versions with and without ammonium were studied, as different diameters (from 9.5 nm to 30 nm). These nanotubes were shown to be non-toxic to both THP-1 cells and human primary monocytes and to induce the production of chemokines in those cells (IL-1 β , IL-6, TNF- α and IL-10) [29,35]. These chemokines are involved in many processes, namely recruitment of T cells to infection sites and inflammation. Interestingly, CNT of low diameter (9.5 nm) and lacking the ammonium group failed in activating the pathways above mentioned and also in inducing production of chemokines in monocytes [29]. These studies suggest that CNT are able to induce an innate immune response dependent both on their functionalization type and size. Macrophages derived from THP-1 cells presented the same phenotype as just described for monocytes [31].

Antigen presentation is a process crucial to mount an immune response against foreign antigens or tumor antigens. Professional antigen presenting cells (APC), such as dendritic cells or macrophages, will uptake a source of antigen (a microorganism, an infected or tumor cell, or a vaccine carrier, such as CNT), degrade it and present it to T cells through the surface complexes MHC class I or class II. While MHC class I induces a more cytotoxic CD8⁺ T cell response, MHC class II shifts the immune response to helper CD4⁺ T cells which will promote an antibody based response [28]. CNT activate both MHC class I and class II, the latter to a greater extent [30,36], suggesting they will induce preferably a humoral response. CNT carrying peptides of immunogens of some pathogens or tumors have been shown to be immunogenic and protective in experimental animal models. CNT containing either peptides for a B cell epitope from the foot-and-mouth disease virus (FMDV), which causes huge economic losses in the cattle industry [37], or the N-terminal (residues 21–42) of *Plasmodium vivax* Apical Membrane Antigen-1 (AMA-1, a micronemal protein highly conserved amongst *Plasmodium* species, the causative agent of Malaria [28,38]), induced a high titer of antibodies [23,39]. Further tests confirmed that the antibodies generated were specific for the regions present in the CNT. They were able to fully neutralize the FMDV infection in vitro and some of the animals immunized with the *Plasmodium vivax* AMA-1 N-terminal were fully protected against challenge with a murine plasmodial species, *Plasmodium berghei*, both sharing a high homology for that AMA-1 region [23,39]. A very recent work showed that a peptide of Wilm's tumor protein 1 (WT1), a kidney cancer, conjugated to SWNT is rapidly internalized by

human APC, namely dendritic cells and macrophages [30]. The SWNT conjugated WT1 peptides were internalized through a macropinocytosis mechanism, known to uptake several macromolecular antigens in immature dendritic cells. They were found to co-localize in lysosomal compartments, which lead to the degradation of antigens and promote their presentation to the immune system through MHC class II molecules. Both conjugated and unconjugated WT1 peptide elicited a similar CD4⁺ T cell interferon- γ (IFN- γ) dependent response in human lymphocytes. However, SWNT conjugated peptides induced a much higher humoral response in Balb/c mice when TiterMax, a commercial adjuvant, was used. This is of particular importance, since tumor self-antigens usually induce poor immune responses.

As mentioned above, CNT can also activate MHC class I receptors [36], which would induce a cytotoxic, Th1 biased cell response, characterized by the production of cytokines such as IFN- γ , TNF- α and IL-12. Although pristine CNT do not induce a CD8⁺ T cell response, there is indirect evidence that functionalized, conjugated SWNT and MWNT originate such response. Zeinali and colleagues tested SWNT coated with tuberculin purified protein derivative (PPD), a mixture of antigens derived from a culture of *Mycobacterium tuberculosis*, the pathogen responsible for tuberculosis [40]. Balb/c mice were immunized with tuberculin PPD, either free or bound to SWNT, receiving two immunizations, 2-weeks apart. Two groups were immunized with free tuberculin PPD, one adjuvanted with complete Freund's adjuvant (CFA) while the other received no adjuvant. The authors did not assess protection against challenge with an infectious bacillus, but compared the cytokine profile induced by the immunization with tuberculin, free or attached to SWNT, with the one elicited by immunization with BCG (a tuberculosis vaccine based on attenuated *Mycobacterium bovis*). The levels of IFN- γ , IL-12, IL-5 and IL-10 were measured in the cultures of splenocytes isolated from the immunized mice. Both groups immunized with BCG and tuberculin PPD bound to SWNT had significantly higher levels of IFN- γ when compared to mice immunized with free tuberculin, adjuvanted or not with CFA. Conversely, the latter groups had significantly higher levels of Th2-type cytokines (IL-5 and IL-10) than the BCG and SWNT immunized groups. Another work shows evidence of cytotoxic T cells induced by CNT. Meng and colleagues conjugated proteins from cellular lysates derived from the murine H22 liver cancer to previously oxidized MWNT [41]. Balb/c mice immunized with a tumor cell vaccine (TCV) based on the H22 liver cancer cells and with MWNT containing proteins of the same cells had a higher cure rate than those immunized with just TCV or TCV together with non-conjugated nanotubes. Also, immunized mice were protected upon further challenge with H22 liver cancer,

never developing cancer unlike control mice. Furthermore, the protection observed was specific, since mice challenged with a different tumor cell line were not protected. Moreover, lymphocytes isolated from immunized mice targeted H22 liver cells, but not the other tumor cell line.

Overall, more work is required to fully understand the action of CNT on the immune system. However, the works described above show clearly that CNT activate the innate immune response, as observed by the transcription of genes involved in several pathways of inflammation, response to infection and vaccination and release of chemokines which will attract APC and consequent mount an adaptive immune response. Macrophages and dendritic cells process efficiently peptides incorporated in CNT and present them in MHC Class I and II, promoting preferably a humoral response against antigens conjugated to them, but also a cellular immune response. CNT were not immunogenic by themselves, since no specific immune response for them was observed [29,30], indicating they can be used when regimens of multiple immunizations are required without risk of losing their stimulatory properties.

Carbon nanotubes as potential adjuvants

Many vaccines use adjuvants, substances that enhance the immunogenic potential of the immunization strategy, without inducing an immune response per se [28]. As described above, CNT stimulate the innate immune system, therefore having inherent adjuvant properties [29,31]. Another work showed that, when administration of embryonic stem cells (ESC) is adjuvanted with MWNT, the therapeutic effect of the former in a colon cancer C57Bl/6 mouse model is enhanced [42], promoting a decrease in the tumor volumes and an increase in cytotoxic CD8⁺ T cells and Th1-type cytokines, such as IFN- γ and IL-2.

It has been suggested that SWNT conjugated with unmethylated CpG DNA motifs can be used as an adjuvant in vaccines [43]. These DNA motifs can be considered a danger signal by the immune system, recognized by Toll-like receptor 9 (TLR-9), an endocytic receptor, and confer protection against several intracellular pathogens and tumors by improving the immune response against them. However, the action of the sole CpG DNA motifs is short-lived and requires administration of high and constant doses, due to the fact both CpG and cellular membranes have a negative charge, impairing the uptake of the former by the cells. Bianco and colleagues conjugated a CpG motif in SWNT, improving their immunostimulatory properties. When incubated with mouse splenocytes, CpG 1668 conjugated to SWNT leads to a decrease in the production of IL-6, compared with the non-conjugated form. Since this cytokine is

pro-inflammatory, the authors believe administration of CpG conjugated with SWNT would be less toxic than using the CpG alone. Also, the SWNT would compensate for the negative charge of the CpG, facilitating its entry in the cells. Another work showed that CpG conjugated with functionalized SWNTs (CpG-SWNT) were avidly internalized by immune cells in a mouse brain tumor (gliomas) model [34]. Unlike the previous work, a pro-inflammatory cytokine response was observed, as shown by the increase in the production of IL-12 and TNF- α by monocytes which suffered uptake of CpG-SWNT. NK cells, macrophages and microglia (APC resident in brain vasculature) also readily uptake CpG-SWNT, resulting in eradication of gliomas in more than 50% of the animals and long lasting immunity when cured mice were re-challenged with homologous tumor. Protection was dependent on NK and CD8⁺ T cells. Neither administration of CpG alone nor CpG co-administrated with unconjugated CNT mimicked these results. This work illustrates the potential of CNT to deliver adjuvant molecules (in this case, CpG) in a mouse model of gliomas, clearly contributing to clearance and immunization against this tumor, and suggests the use of this strategy as adjuvants either for other tumors or even for intracellular pathogens.

Toxicity of carbon nanotubes

One of the main concerns regarding the use of CNT in vaccines and other human therapeutic or prophylactic interventions is their toxicity. Due to their small size, they can spread within the organism, reaching several crucial sites, which represents both a concern in terms of cytotoxicity and an opportunity in terms of their potential as vaccine carriers. Several evidences show that CNT can be toxic both *in vitro* and *in vivo*, inducing the production of cytotoxic reactive oxygen species (ROS), cell apoptosis and necrosis [24,44,45]. It has also been shown that CNT can bind to several plasma proteins and can activate the innate immune system complement pathways, leading to inflammation [46]. Although this could have an adjuvant effect, in excess it could be deleterious for the host if high levels of inflammation are induced. However, the toxicity of CNT is dependent of several factors, namely the dose used and their solubility in water. Unsurprisingly, the higher the dose, the more pronounced are the toxic effects. It could in fact be argued that for most compounds, very high doses induce a toxic effect. Furthermore, CNT that are functionalized in order to be more soluble in water are less toxic [16]. In particular, PEG or PEG-phospholipid dispersed CNT were shown to be well tolerated in biological systems both *in vivo* and *in vitro* [44,47]. Functionalized CNT are also more biocompatible in mice, being slowly excreted through urine and feces, therefore being less

prone to potential toxic effects caused by accumulation, and are also more hemocompatible, since hematological analyses show no major differences between naïve animals and mice subjected to CNT [44,45,48,49]. Additionally, an accurate purification of CNT to ensure toxic chemicals or metals used during their production and in the functionalization steps are effectively removed, also reduces their toxicity. However, this process is very laborious and complex. Overall, the actual level of toxicity of CNT, as shown in the literature and assessed by our own experience, remains controversial and studies with established toxicology models have still to be optimized and implemented [16].

It is important to note that the majority of the works mentioned above, do not report any adverse effect of CNT in cells or mice, including those that specifically addressed the issue of toxicity. For the tuberculin PPD study, Zeinali and colleagues showed that although SWNT generate ROS in Balb/c mice splenocytes, it had no effect in the cell viability [40]. Also, nitric oxide, an end product of inducible nitric oxide synthase, which would be an indication of an inflammatory response, could not be detected in the supernatants of mouse macrophage cultures previously incubated with SWNT alone or conjugated with PPD. Moreover, immature dendritic cells incubated with doses of SWNT up to 100 $\mu\text{g}/\text{mL}$ were not affected in their functionality and viability [30].

Toxicity also varies according to the different routes of administration [44,45]. Non-dispersed CNT tend to form larger aggregates and therefore are more toxic. Works with pristine CNT administered through the respiratory tract in animal models show a high degree of inflammation, granuloma formation and obstruction of the upper airways [31,45,50]. Even functionalized CNT seem to induce toxicity in lungs in rats [51]. Although some strategies were shown to circumvent the issue of toxicity following intranasal administration, such as CN_x NT, nanotubes doped with nitrogen groups [52], the risk of mechanical obstruction may persist, making this route less appealing for immunization with CNT. Administration of pristine CNT subcutaneously and intraperitoneally in mice also led to undesired toxic effects, such as granuloma formation or inflammation [45,53,54]. Conversely, when CNT which suffered several functionalization methods (e.g., PEG, amino acids, etc.) were injected in mice using the same routes, only in some cases low levels of inflammation were observed [23,30,39-41,44,55,56]. Oral administration of both pristine and functionalized CNT did not induce toxicity in mice [44,45,53,57,58].

An useful overview of the concerns related to toxicity in the biomedical applications of nanotubes and the related regulatory issues has been elegantly discussed elsewhere [59].

Conclusions

It is clear that CNT have a high potential for delivery of antigens and to be considered as a novel vaccine platform for both infectious diseases and cancer, due to the promising results mentioned above. Their nanometric dimension allows them to be easily internalized by cells. They have a large inner volume compared to their linear dimensions and biomolecules can be easily immobilized on their outer surface. Such confers them an advantage to be used as nanocarriers for controlled and targeted drug delivery [59]. As stated, further studies regarding the toxicity of these nanostructures are required and new approaches are being undertaken to enhance the capacity of cells to degrade CNT, such as shortening of CNT [4]. Their immunization potential can be improved upon confirmation that the conjugated epitopes maintain their correct conformation, a key aspect for an efficient immune response elicited against them [60]. Furthermore, the recent discovery that certain enzymes such as myeloperoxidases can degrade CNT leads to believe that, if antigens are encapsulated within these nanostructures, they would be more protected from external factors [61]. After cellular internalization and degradation of CNT, antigens could still be efficiently presented. Functionalized CNT can be administered using routes commonly used in vaccination, such as subcutaneous and oral without inducing severe, undesired toxic effects [23,30,39-41,44,55-57] which strengthens the possibility of their usefulness as immunization strategies.

Nevertheless, the promise of CNT to revolutionize the field of biomedical applications is yet unmet. In fact, as clearly described by Kostarelos and colleagues "The use of CNT in medicine is now at the crossroads between a proof-of-principle concept and an established preclinical candidate for a variety of therapeutic and diagnostic applications. Progress towards clinical trials will depend on the outcomes of efficacy and toxicology studies, which will provide the necessary risk-to-benefit assessments for carbon nanotube based materials" [16]. Whether CNT will meet these expectations is at the moment difficult to predict, but everything indicates most probably they will. Not long ago, the use of CNT in material science applications was even regarded as some science fiction tool [62]. However, nowadays, yarns of nanotubes are routinely produced in an up scalable process [63], and can be woven to form ropes and fabrics with exceptional properties [64]. Current and further research will definitely unravel the potential of CNT to be used in vaccination approaches against infectious diseases and cancer.

Abbreviations

CNT: Carbon nanotubes; SWNT: Single walled carbon nanotubes; MWNT: Multiwall carbon nanotubes; DNA: Deoxyribonucleic acid; PEG: Polyethylene glycol; IFN- γ : Interferon- γ ; IL: Interleukin; TNF- α : Tumor

necrosis factor α ; NF- κ B: Nuclear factor kappa-light-chain-enhancer of activated B cells; FMDV: Foot-and-mouth disease virus; CFA: Complete Freund's Adjuvant; iCFA: Incomplete Freund's Adjuvant; NK: Natural killer cells; AMA-1: Apical membrane antigen-1; BCG: Bacillus calmette-guerin; PPD: Purified protein derivative; ESC: Embryonic stem cells; TCV: Tumor cell vaccine; WT1: Wilm's tumor protein 1; APC: Antigen presenting cell; ROS: Reactive oxygen species.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

RG and BD participated equally in the drafting and writing of this manuscript. Both authors read and approved the final manuscript.

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