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Achievements, challenges and chances

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Application of carbon nanotubes in cancer vaccines: achievements, challenges and chances

*Hatem A. F. M. Hassan¹, Sandra S. Diebold², Lesley A. Smyth³, Adam A. Walters¹,
Giovanna Lombardi⁴, Khuloud T. Al-Jamal^{1*}*

¹Institute of Pharmaceutical Science, School of Cancer and Pharmaceutical Science, Faculty of Life Sciences & Medicine, King's College London, Franklin-Wilkins Building, London SE1 9NH, United Kingdom

²Biotherapeutics Division, National Institute for Biological Standards and Control (NIBSC), Blanche Lane, South Mimms, Potters Bar, Hertfordshire EN6 3QG, UK.

³School of Health, Sport and Biosciences, University of East London, Stratford Campus, Water Lane, London E15 4LZ, UK.

⁴Immunoregulation Laboratory, MRC Centre for Transplantation, King's College London, Guy's Hospital, London SE1 9RT, United Kingdom

*Corresponding address:

Prof. Khuloud T. Al-Jamal

Chair of Drug Delivery & Nanomedicine

School of Cancer and Pharmaceutical Science, King's College London

Franklin-Wilkins Building

150 Stamford Street

London SE1 9NH; Tel: 020 7848 4525; Email: Khuloud.al-jamal@kcl.ac.uk

Abstract

Tumour-specific, immuno-based therapeutic interventions can be considered as safe and effective approaches for cancer therapy. Exploitation of nano-vaccinology to intensify the cancer vaccine potency may overcome the need for administration of high vaccine doses or additional adjuvants and therefore could be a more efficient approach. Carbon nanotube (CNT) can be described as carbon sheet(s) rolled up into a cylinder that is nanometers wide and nanometers to micrometers long. Stemming from the observed capacities of CNTs to enter various types of cells *via* diversified mechanisms utilising energy-dependent and/or passive routes of cell uptake, the use of CNTs for the delivery of therapeutic agents has drawn increasing interests over the last decade. Here we review the previous studies that demonstrated the possible benefits of these cylindrical nano-vectors as cancer vaccine delivery systems as well as the obstacles their clinical application is facing.

Keywords: Cancer immunotherapy, antigen presenting cells, nanomedicine, vaccine delivery, carbon nanotube.

Table of contents

1. Introduction	4
2. Carbon nanotubes (CNTs) as nanocarriers	6
2.1 Spheres vs Tubes vs Sheets as nanocarriers	7
2.2 Mechanisms of CNTs' cellular uptake	8
2.3 CNTs' Biocompatibility <i>in vitro</i>	13
2.3.1 Effect of CNTs' chemical functionalisation	13
2.3.2 Biocompatibility with immune cells	15
2.4 CNTs' biodistribution	16
2.5 CNTs' Biodegradability	19
3. Functionalised CNTs as cancer vaccine delivery system	23
3.1 Functionalised CNTs as delivery vector for tumour-derived antigen	24
3.2 Functionalised CNTs as delivery vector for adjuvants	27
3.3 Functionalised CNTs as delivery vector for both tumour-derived antigen and adjuvants	29
4. Future perspective of CNTs as vaccine delivery systems	32
5. Conclusions	36

1. Introduction

Conventional treatments for cancer include surgery, chemotherapy and radiotherapy. Surgical operations can be associated with complications arising from damaging the tissues surrounding the tumour site. Radiotherapy, which relies on the application of ionising radiation to the tumour sites, leading to DNA damage and inhibition of cancer cell proliferation, can also affect the division of normal cells such as blood forming cells in bone marrow [1, 2]. In addition, cancer surgery and radiotherapy are only efficient in treating localised tumour. In cases where cancer cells have metastasised, i.e. gained access to sites distal to the primary tumour, chemotherapeutic drugs, e.g. cisplatin, are administered to slow down cancer cell proliferation and prolong patient survival [3]. Chemotherapeutic agents are typically non-specific and can also inhibit the proliferation of normal cells leading to serious side effects such as myelosuppression and immunosuppression [2, 4], which can limit the administration of higher doses to achieve better efficacy.

The human body is equipped with a number of defensive mechanisms that can be harnessed to fight the cancer cells. In fact, it is hypothesised that the immune system is in a continuous state of cancer immune surveillance [5]. According to this concept, professional antigen presenting cells (APCs) such as the dendritic cells (DCs) sample all tissues for the presence of stress signals such as danger-associated molecular patterns which are upregulated in tumour tissues. The presence of such stress signals activates the innate immune system and the resulting immune response keeps tumour growth in check. Once the cancer cells acquire mutations that let them escape the control by the immune system they acquire the ability to establish tumours. Given this, the use of immunotherapy for cancer has attracted significant interests over the last decades culminating in numerous clinical trials [6].

Cancer vaccines are one such intervention. Like traditional vaccines against infectious disease, cancer vaccines are comprised of cancer cell-derived antigens formulated in such a fashion as to provoke a potent immune response. Typically the antigenic payload of these vaccines are either mutated proteins arising as a direct result or as a byproduct of tumorigenesis (so called neo antigens), proteins which are overexpressed in tumours or proteins which are the result of aberrant expression of embryonic genes in tumours [7-9]. The anti-tumour immune responses elicited by the cancer vaccine is aimed to systemically target the cancer cells throughout the whole body; hence cancer immunotherapy can be used to treat metastatic tumours [10]. Moreover, immunisation with cancer vaccines can induce persistent T cell memory-specific against the tumour cells, providing long-lived protection and prolonged patient survival [11].

Unfortunately, while in preclinical models cancer vaccine have proven efficacious there has been limited progress in the development of human cancer vaccines, with a number of high profile candidates failing to meet their end points in clinical trials [12]. This is, in part due to the failure to overcome tumour-induced immunosuppression [13, 14]. However, there has been a surge of renewed interest in the field since the advent of checkpoint blockade and increased understanding of the immune suppressive tumour micro-environment [15].

It is likely that a successful cancer vaccine will be composed of three components: the antigen, the adjuvant and the delivery vehicle. This regime may or may not be supplemented with a checkpoint inhibitor. Speculating further it may be proposed that cancer vaccines will require novel formulations distinct from formulations previously used for infectious disease (such as alum absorbed antigen) as cancer immunity will primarily be driven by cell-mediated cytotoxic responses rather than antibody-mediated

humoral responses [6]. This review discusses one such formulation; carbon nanotubes in the context of cancer vaccines.

2. Carbon nanotubes (CNTs) as nanocarriers

CNTs are synthetic allotropes of carbon. Allotropy is described as the chemical elements ability to exist in more than one form. Three allotropic forms have been identified for carbon. The natural carbon allotropes include diamond, graphite (several layers of graphene) and amorphous carbon (non-crystalline form of carbon) [16]. The synthetic carbon allotropes that have been discovered include fullerene (sphere of carbon atoms) [17], graphene (single layer of graphite) [18] and CNT (cylinder consisting of rolled graphene layer(s)) (Fig. 1.) [19]. Morphologically, CNTs can be described as cylinders that are nanometers wide and nanometers to micrometers long, consisting of graphene rolled up in the form of single or multiple concentric layer(s) that are referred to as single-walled CNT (SWNT) or multi-walled CNT (MWNT), respectively [20].

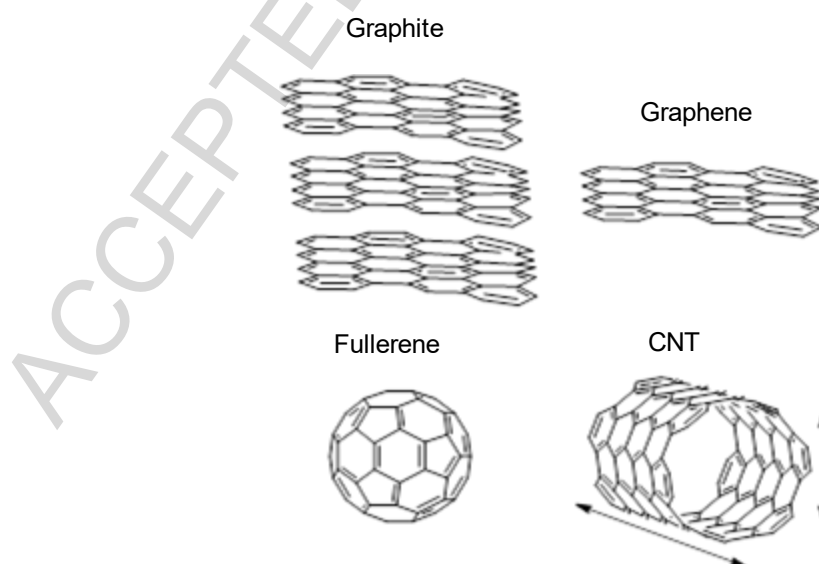


Fig. 1. Different allotropes of carbon.

2.1 Spheres vs Tubes vs Sheets as nanocarriers

In terms of carbonaceous nanomaterials two carriers have been widely employed, namely CNT and the carbon nanosheet Graphene oxide (GO). Unlike most other materials there has been minimal work assessing the spherical form of carbon: fullerenes, as a carrier [21]. Therefore, it is difficult to attribute the observed effects to morphology or material composition when comparing CNT to other carriers. However, morphology dependant behaviour can be observed in other systems. For instance, Trewyn *et al.* compared the cellular uptake of silica nanoparticles that were either tube-shaped (100 nm wide and 600 nm long) or spherical shaped (115 nm in diameter) of comparable surface charges [22]. The tube-shaped silica nanoparticles demonstrated higher uptake by CHO cells or fibroblast cells, *in vitro*, compared to the spherical ones. Similarly, Huang *et al.* have reported that internalisation of rod-shaped silica nanoparticles (100 nm wide and 450 nm long) by the epithelial A375 line *in vitro* was higher compared to the spherical nanoparticles (100 nm in diameter) [23]. Whether the carrier's morphology-dependent cellular uptake affects the biological response induced by the loaded cargo demands future investigations. In light of these findings and hypotheses, comparative studies need to be carried out to investigate the cellular uptake and immunogenicity of CNTs versus spherical nanoparticles, ideally fullerenes but also the extensively studied poly(lactic-co-glycolic acid) (PLGA) nanoparticles and liposomes. The findings of such studies will undoubtedly contribute to the nanovaccinology field and further searches for nano-carriers with optimal morphological properties capable of efficient vaccine delivery.

The other commonly used carbon allotrope is the carbon nanosheet, GO [24-27]. The use of GO, as a cancer vaccine carrier, has been assessed by several groups. Yue *et al.* demonstrated that a subcutaneous injection of GO-OVA in C57BL/6 mice showed

significantly elevated OVA-specific CTL response in comparison to OVA alone [28]. Furthermore, in a thymoma model immunisation of the aforementioned mice with GO-OVA was more efficient than OVA alone in limiting the growth of E.G7-OVA lymphoma cells. Testing the ability to induce protective anti-tumour immunity, Sinha *et al.* have reported that compared to C57BL/6 mice immunised with OVA alone, mice vaccinated with dextran-functionalised GO-OVA showed smaller tumour sizes after challenging with subcutaneous injection of OVA-expressing melanoma B16 cells [29]. These findings presented GO as a competent vaccine nanocarrier. Both CNTs and GO possess attractive properties of being able to incorporate the biomolecules of interest *via* simple surface adsorption. However, the proficiency of these carbon nanosheets (GO) compared to the CNTs in delivering vaccines remains in question. Zhang *et al.* have demonstrated that radiolabelled oxidised MWNTs were taken up in higher amounts compared to radiolabelled GO, by HeLa cells *in vitro* [30]. The degree of cellular uptake is a key, but is not the only, factor determining the intensity of elicited immune response. Proper comparative assessment of CNTs and GO uptake by the APCs and the subsequent impact on the induced immune response will assist future researches on further developing carbon nanocarriers suitable for vaccine delivery.

2.2 Mechanisms of CNTs' cellular uptake

Pristine (unmodified) CNTs are hydrophobic in nature and are thus characterised by their low dispersibility and high tendency to form aggregates in aqueous media. CNT bundle formation is attributed to the non-covalent interactions between the nanotubes such as the π - π stacking that occur between the aromatic rings of adjacent nanotubes. Thereby, it was essential to find chemical approaches that can improve the CNTs biocompatibility by enhancing the CNTs degree of individualisation and dispersibility in physiological fluids. The chemical approaches that have been applied to functionalise

CNTs can be classified into covalent and non-covalent functionalisations [31, 32]. For instance, utilising covalent functionalisation, pristine CNTs sonication in a mixture of acids (e.g., sulphuric and nitric acids [33, 34]) can incorporate carboxylic groups that can be activated using carbodiimide-based approach which can be further derivatised with amine-terminated linkers yielding functionalised CNT with improved hydrophilicity [32]. Relying on non-covalent interactions, Zheng *et al.* have shown that SWNT dispersibility in aqueous solution was improved by sonication with single stranded DNA (ssDNA) [35]. Wang *et al.* have also demonstrated that bovine serum albumin non-covalent conjugation with oxidised MWNTs led to an enhanced dispersibility in phosphate-buffered saline [36].

In order to assess the possible benefits that can be obtained from using CNTs as therapeutics delivery vector, it was important to study the mechanisms by which these nanoscopic scaffolds can enter the cells. CNTs have demonstrated high ability to enter various types of cells [37-39]. It has been previously reported that polymeric spherical nanoparticles, e.g. PLGA nanoparticles, mainly utilise energy-dependent mechanisms of cellular uptake rather than energy-independent ones [23, 40, 41]. However, there is good data to suggest the efficiency and uptake route of CNTs is highly dependent on morphology and surface chemistry.

For instance, Shi Kam *et al.* showed that the cellular uptake of fluorescent labelled streptavidin protein, by HL60 leukaemia cells, was improved by its conjugation to oxidised SWNTs [42, 43]. The uptake of the SWNT-streptavidin conjugate was reduced when cells were placed at 4 °C, suggesting that the internalisation of SWNT was carried out *via* endocytosis, which is an energy-dependent mechanism. In addition, staining the endosomes with a fluorescent marker followed by confocal microscopy revealed the presence of SWNT-streptavidin in the endocytic vesicles of these cells, which further

supported the proposed endocytosis mechanism. Relying on the detection of SWNTs intrinsic near infra-red fluorescence to assess the cellular uptake, Cherukuri *et al.* have shown that the uptake of pristine SWNTs, dispersed in Pluronic surfactant, by mouse macrophage cells *in vitro* was dramatically reduced by incubating the cells at 27 °C rather than 37 °C [44]. This finding also suggested an endocytosis-dependent mechanism for SWNTs uptake.

On the other hand, other studies have suggested that the cellular uptake of CNTs is mainly achieved *via* passive diffusion through the cell membrane. Pantarotto *et al.* have demonstrated that amine-functionalised SWNTs improved the internalisation of fluorescent labelled peptide by human 3T6 fibroblasts incubated at 37 °C, as determined by epifluorescence microscopy that also revealed the presence of the SWNTs in the nucleus [45]. Inhibiting the metabolic functions of cell by incubation at 4 °C or treatment with sodium azide did not affect the cellular uptake of the SWNTs, which suggested an energy-independent mechanism of cell uptake. The CNT's ability to penetrate through the cell membrane, like nano-needles, could be explained by the nanosyringe mechanism theoretically simulated by Lopez *et al.* for nanotubes insertion into the lipid bilayer [46]. Molecular dynamics presented by Lopez *et al.* showed that hydrophobic nanotube with hydrophilic tips firstly adsorbs, in a horizontal plane, onto a model membrane [46]. Partial insertion of the nanotube into the model bilayer is then followed by spontaneous change in the nanotube orientation from the horizontal into the vertical alignment, forming a transmembrane pore-like orientation through the bilayer. Insertion into the membrane bilayer could be then followed by translocation to the intracellular compartments as reported by Pantarotto *et al.* In another study, further investigation of the energy-independent CNT uptake was carried out, and confocal microscopy images revealed the internalisation of fluorescent labelled and amine-functionalised MWNTs by

fungus (*Saccharomyces Cerevisiae*) and yeast cells (*Cryptococcus neoformans*) [47]. The functionalised MWNTs internalisation by these prokaryotic cells (which normally lack the ability to carry out active processes such as endocytosis) highlighted the MWNTs' utilisation of mechanisms other than endocytosis for cell entry. In addition, it was shown that Jurkat leukaemic T cells incubation with the functionalised MWNTs at 4 °C in the presence of sodium azide did not inhibit the cellular uptake of MWNTs, which confirmed the involvement of passive cellular uptake mechanism. The contradicting mechanisms of CNTs cellular uptake reported by different authors could be attributed to the properties of the CNTs used in these studies such as the CNTs' degree of individualisation and dispersibility in the cell culture media, which is highly dependent on the CNTs' functionalisation density.

Mu *et al.* have studied the uptake of functionalised MWNTs by HEK293 epithelial cells using TEM [48]. In this study, single MWNT were imaged penetrating the cell membrane, while MWNTs bundles were found intracellularly surrounded by endosomal membrane. Single MWNT released from the MWNTs bundle entrapped in endosomes were also imaged while penetrating the endosomal membrane, to enter the cytosol.

Collectively, from these studies it could be concluded that CNTs can access the intracellular compartments *via* more than one mechanism of cell entry. This could be attributed to the length to width ratio of the CNTs that allows the, nanoneedle-shaped, CNTs to passively penetrate the cell membrane utilising the hypothesised and experimentally demonstrated nanosyringe mechanism, in addition to the active endocytosis mechanism. The role of uptake cannot be understated as the ultimate goal of cancer vaccines is to induce a tumour-specific cytotoxic and memory CD8⁺ T cells responses, capable of eradicating the established tumours and providing long-term protection, respectively [12]. Promoting antigen translocation to the DC's cytosol, where

proteasomal processing occurs, could enhance antigen cross-presentation and, subsequently, the induction of antigen-specific CD8⁺ T cell response [49]. The fact that CNTs can passively diffuse through the cell membrane and reach the cytosol or leak through the endosomes into the cytosol following internalisation *via* endocytosis could suggest that CNTs are qualified, as delivery vector, to translocate their loaded antigen to the cytosolic compartments (**Fig. 2.**) [45, 47, 48]. The CNTs ability to penetrate the cell membrane might be assigned to their nanoneedle-like structure which arises from their high aspect ratio (length to width ratio) [46]. Nevertheless, endosomal membrane disruption associated with CNTs endosomal escape could induce cell damage [50, 51]. Membrane disruption could activate NLRP3 inflammasome and consequently induce pyroptotic cell death [50, 51], and is a concern that should not be overlooked on designing a CNT-based vaccine delivery system.

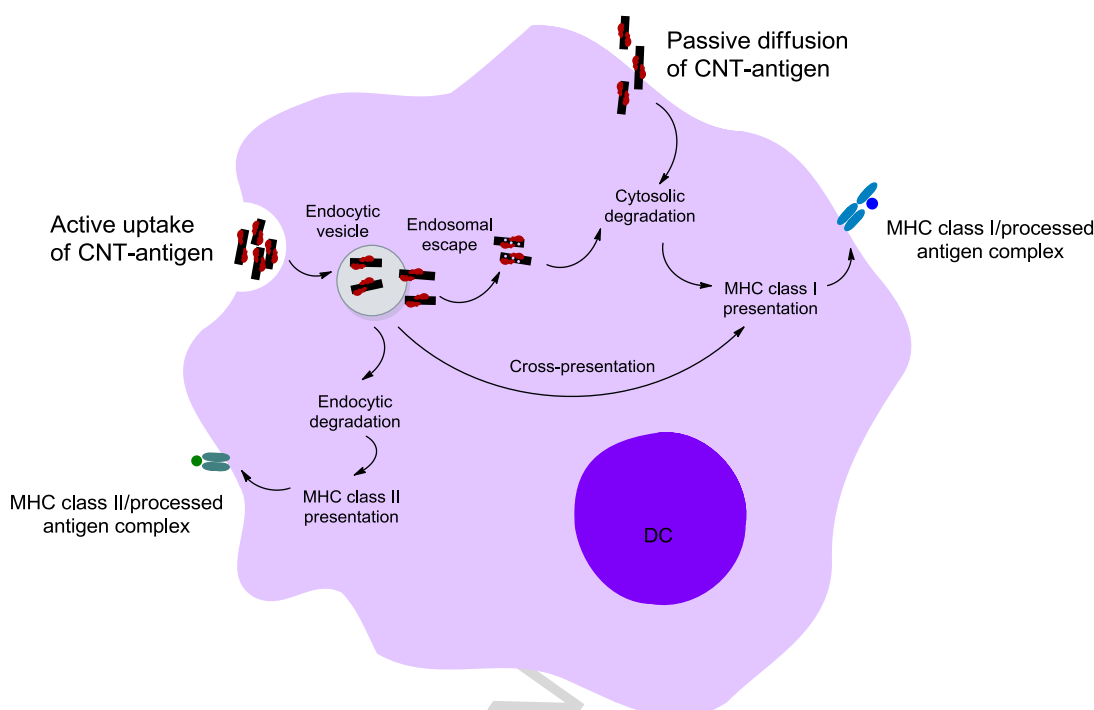


Fig. 2. Proposed pathways for MHC presentation of CNTs-delivered antigens. CNTs could deliver the incorporated antigen to the cytosol of the DCs, *via* two proposed routes, where degradation by proteasome and subsequent MHC class I presentation occur. The ability of CNT-antigen conjugates to passively diffuse through the cell membrane could directly deliver the loaded antigen to the cytosol. Alternatively, following the active uptake of CNT-antigen conjugates by DCs and the subsequent endosomal escape; the incorporated antigen could gain entry into the cytosol. In addition, overcoming the need for endosomal escape, antigenic fragments yielded from antigens processed in the endosomes by endosomal proteases could be loaded onto the MHC class I molecules recycled from the plasma membrane. Furthermore, professional cross-priming DCs could translocate endocytosed antigenic cargo to the MHC class I pathway *via* the utilisation of cross-presentation mechanism. Lysosomal degradation of CNTs-delivered antigens that fail to escape the endosomes could be followed by MHC class II presentation.

2.3 CNTs' Biocompatibility *in vitro*

2.3.1 Effect of CNTs' chemical functionalisation

Residual transition metal catalysts such as iron, cobalt or nickel contained in the pristine CNTs can catalyse the intracellular formation of free radicals and oxidative stress leading to cytotoxic effects. For instance, treating HEK293 human kidney embryo cells

with pristine SWNTs induced cell apoptosis and reduced cell proliferation [52], additionally incubating Calu-3 human epithelial cells with pristine MWNTs significantly reduced cell viability [53]. However, chemically functionalised CNTs have shown better biocompatibility profiles compared to the pristine material. This could be attributed to the fact that exposing pristine CNTs to chemical reactions followed by successive washing in organic solvents with the aid of bath sonication help in removing metal catalysts adsorbed onto the CNTs wall. In addition, chemical reactions, such as bath sonication-assisted acid oxidation, that generate surface defects onto the CNTs help in removing trapped metal catalysts [54].

Coccini *et al.* have shown that acid-oxidised MWNTs possessed lower metal content and exerted minor effects on the cell viability compared to the pristine MWNTs following incubation with the epithelial A549 cells at 1 $\mu\text{g/ml}$ [55]. However, increasing the oxidised MWNT concentration in the culture media was accompanied by dramatic decrease in the A549 cells viability. Vuković *et al.* have reported that treating fibroblast L929 cells with pristine MWNTs at concentrations ranging 3–12.5 $\mu\text{g/ml}$ significantly reduced the cell proliferation, while incubation with carboxylic or amine-functionalised MWNTs did not show this effect [56]. Despite this, the proliferation of the L929 cells incubated with the functionalised MWNTs at higher concentrations (25–100 $\mu\text{g/ml}$) was significantly lower than the untreated cells. Incubating human epidermal keratinocytes with acid-functionalised SWNTs also showed a dose-dependent reduction in cell viability and increased production of the pro-inflammatory cytokine IL-8 [57]. This observation could be attributed to the rupture of CNT-contained endosomes that led to NLRP3 inflammasome activation and pyroptosis [50, 51].

It could be suggested that although chemical functionalisation can improve the CNTs purity and biocompatibility, the associated increase in CNTs individualisation increased

their cellular uptake thus the possibility of causing cytotoxic effects with increased dosage. In a similar fashion, Li *et al.* have demonstrated that as the positivity of chemically functionalised MWNTs was increased (by manipulating the surface chemistry), the cellular uptake by THP-1 cells (monocytic cell line) and BEAS-2B cells (bronchial epithelial cell line) was enhanced causing production of pro-inflammatory cytokines [58].

2.3.2 Biocompatibility with immune cells

Cytotoxic effects of CNTs on immune cells have been investigated in various studies. Using TEM imaging, it was demonstrated that carboxylated SWNTs formed less intracellular aggregates following incubation with human monocyte-derived macrophages and exerted lower effects on the cell viability than pristine SWNTs [59]. Treating murine RAW 264.7 macrophages with pristine SWNTs (26 wt% of iron) led to significant depletion of glutathione (oxidative stress biomarker) compared to treatment with carboxylated SWNTs (0.23 wt% of iron) [60]. The higher iron content of pristine SWNT than carboxylated SWNTs also led to significant increase in the formation of intracellular reactive oxygen species following incubation with rat NR8383 macrophages [61]. The length of the CNT could also determine the CNTs' biocompatibility. LPS-primed, human primary macrophages treated with long pristine MWNT (~13 μm) showed higher production of the NLRP3 inflammasome-mediated inflammatory cytokine IL-1 β than shorter pristine MWNTs (1-10 μm) treated macrophages [51, 62].

Wang *et al.* have shown that treating human monocyte-derived DCs with carboxylated-MWNTs at 10-100 $\mu\text{g/ml}$ for 48 hr was not associated with a significant decrease in cell viability [63]. In addition, the carboxylated-MWNT treatment did not increase the DCs expression of the CD80 or CD86 co-stimulatory molecules that suggested lack of MWNTs adjuvanticity. However, cytokines production by MWNTs-

treated DCs was not evaluated in this study. Dumortier *et al.* have demonstrated that culturing mice-derived B or T lymphocytes in the presence of SWNTs functionalised using 1,3-dipolar cycloaddition reaction did not induce cell death, provoke cell proliferation or stimulate IFN- γ production [64]. Nevertheless, SWNTs functionalised *via* acid-oxidation and amide coupling reactions that exhibited lower aqueous dispersibility stimulated the production of TNF- α and IL-6 by macrophages *in vitro*. Although this study highlighted the effect of SWNTs surface chemistry, and consequently the aqueous dispersibility, on the cytokine production by immune cells *in vitro*, it would be also useful to investigate the effect of different sized SWNTs.

Pescatori *et al.* have also reported that incubating carboxylated or amine-functionalised MWNTs with Jurkat T cell line or THP-1 monocytic cell line did not induce cell apoptosis [65]. The uptake of the functionalised MWNTs by the THP-1 monocytic cells, but not the Jurkat T cells, increased the production of IL-6 and TNF- α pro-inflammatory cytokines. On the other hand, Medepalli *et al.* have found that DNA-functionalised SWNTs did not alter the cell phenotypes or activation markers expression following the incubation with human blood-derived monocytes or lymphocytes [66]. Such discrepancy could be attributed to the CNTs' functionalisation density, the used doses and incubation time.

These studies, therefore, suggested that the increased purity of functionalised CNTs could be accompanied by improved cytocompatibility. However, functionalised CNTs could still exert dose-dependent cytotoxic effects.

2.4 CNTs' biodistribution

Biodistribution and clearance of the CNTs are considered among the main obstacles clinical application of CNTs is facing. The utilisation of CNTs for the delivery of

therapeutic agents demanded studying their biodistribution to determine their organ accumulation and toxicity following systemic administration. Yang *et al.* have demonstrated that pristine SWNTs intravenously injected to KM mice were distributed mainly to liver, spleen and lung 24 hr post injection [67]. The pristine SWNTs were retained in these organs at high levels over a period of 28 days post injection. In addition, the pristine SWNTs were un-detectable in the urine or feces samples collected from injected mice, which further indicated the retention and accumulation of the injected pristine SWNTs in the mentioned organs.

We have shown that the functionalisation density of MWNTs affected their biodistribution and excretion following intravenous injection in BALB/c mice [68]. On comparing three types of ^{111}In -radiolabelled MWNTs that were amine-functionalised to varying degree, the highest bladder accumulation and lowest liver retention detected at 0.5 hr and 24 hr post injection, respectively, were demonstrated by the MWNT that possessed the highest content of amine moieties. Thus, it was suggested that increasing the amine-functionalisation density of MWNTs assisted their individualisation, hence glomerular filtration and renal clearance. However, other factors that are, theoretically, expected to affect the MWNTs biodistribution such as frequency of administration and injected doses were not studied.

Liu *et al.* used Raman spectroscopy to qualitatively and quantitatively assess the long-term fate of SWNTs functionalised with polyethylene glycol (PEG) of varying chain length intravenously administered to BALB/c mice [69]. Raman spectroscopic analysis of *ex vivo* tissues isolated 1 day post-injection of 20 μg SWNTs/mouse showed retention of SWNTs-PEG mainly in liver and spleen. The retained SWNTs-PEG, especially if functionalised with a long PEG chain, were almost cleared from the liver and spleen 3 months after injection with no apparent toxic effects detected in histology

specimens and blood chemistry. Administration of higher doses (100 µg/mouse), presumably due to improvement in signal-to-noise-ratio, provided evidence of SWNTs presence in the bladder and feces at 24 hr post-injection suggesting elimination *via* the renal and biliary routes. On the other hand, the biodistribution and clearance of the injected 100 µg SWNTs over longer period of time, e.g. 1 and 3 months, were not evaluated.

Schipper *et al.* have reported that on monitoring the blood count of nude mice over a period of 4 months after the intravenous injection of PEG-functionalised SWNTs, no significant differences were detected between the naïve and SWNT-injected mice groups [70]. Histological analysis of tissues from the organs excised 4 months post SWNT injection showed the presence of dark aggregates that were confirmed by Raman spectroscopy to be SWNTs in the liver and spleen macrophages with no obvious pathological features. This observation comes despite the fact that mice were injected with SWNTs two times (on day 0 and 7), the injected dose of SWNTs was 17 µg/mouse. It is quite probably that higher doses of SWNTs as nanocarriers will be required and this could be addressed in future work using a similar model.

Guo *et al.* studied the biodistribution and excretion of radiolabelled glucosamine-functionalised MWNTs following intraperitoneal administration to mice [71]. Tracing the radioactivity 1 hr post injection revealed the distribution of MWNTs-glucosamine to the main organs and almost complete clearance after 24 hr with less than 70% of the total initial radioactivity excreted in urine and feces. However, free radiolabelled glucosamine released from the functionalised MWNTs *in vivo* could provide misleading data about the MWNTs biodistribution, thus a more reliable method, e.g. detection of MWNTs Raman signals, could have been applied to further support the presented conclusion.

Meng *et al.* investigated the organ toxicity and immunological reactions induced following subcutaneous administration of 1 mg of carboxylated MWNTs to BALB/c mice [72]. Histological analysis of heart, liver, kidney and spleen excised from the mice over a period of 2 to 90 days post MWNTs injection revealed normal histology with no apparent accumulation of MWNTs. However, histological examination of axillary lymph nodes excised 30 days post injection showed accumulation of MWNTs that increased and then decreased 60 and 90 days, respectively. Nevertheless, the administered MWNTs were not completely eliminated and the study did not investigate the fate of the accumulated MWNTs. Levels of pro-inflammatory cytokines such as TNF- α and IL-17 were detected at a higher level in the sera of the MWNT-injected mice compared to naïve mice 2 days post injection, however, the cytokine levels returned to normal 7 days post injection [72].

In light of the research gaps highlighted in the mentioned studies, future studies could provide more conclusive assessment of the CNTs by considering critical factors such as administered doses, frequency of administration, reliability of the methods applied to track the injected CNTs and long-term tracking following administration.

2.5 CNTs' Biodegradability

Once the loaded cargo is delivered to its intended intracellular target, CNTs need to be eliminated from the cells. To this end, there have been growing interests in studying the CNTs biodegradability and susceptibility to enzymatic degradation. Allen *et al.* have shown that oxidised SWNTs incubation with the plant-derived horseradish peroxidase enzyme at 4 °C in the presence of hydrogen peroxide (H₂O₂) led to length shortening and then complete loss of the tubular structure of the SWNTs, as revealed by TEM imaging, over a period of 12 weeks [73]. It was suggested that the SWNTs were subjected to

biodegradation that was further demonstrated using other methods such as mass spectrometry. Despite the harsh conditions applied, complete biodegradation was not observed as the TEM images revealed the presence of the carbonaceous material at the end of the 12 weeks. In addition, the biodegradation of the SWNTs at conditions resembling the intracellular environment was not evaluated. The described mechanism behind this degradation relies on the interaction of the peroxidase enzyme with H_2O_2 to generate potent oxidising intermediates that oxidise the CNTs into aromatic oxidised fragments and eventually carbon dioxide.

Kagan *et al.* have demonstrated that incubating human neutrophil-derived myeloperoxidase enzyme and H_2O_2 with a suspension of oxidised SWNTs in buffered saline changed the dark SWNTs suspension into a translucent solution after 24 hr [74]. The SWNT biodegradation was assessed by the complete disappearance of the tubular SWNT structure in TEM images and the distortion of the characteristic SWNT Raman signals. Raman spectroscopic analysis of SWNTs internalised by neutrophils *in vitro* was also used to assess intracellular biodegradation. However, the disappearance of the SWNTs characteristic tubular structure or Raman signals cannot be considered as the carbonaceous material persists. The study investigated the biocompatibility of the carbonaceous material by demonstrating that administration of 40 μ g of the biodegraded SWNTs *via* pharyngeal aspiration was not associated with acute pro-inflammatory response in mice. Nevertheless, factors such as other administration routes, higher doses and possibility of chronic toxicity were not studied. In a similar manner, the same group has also studied the biodegradation of oxidised SWNTs following incubation with eosinophil peroxidase enzyme and H_2O_2 or internalisation by murine-derived eosinophils *in vitro* [75]. It would have been interesting to assess the biodegradation of various SWNTs doses following incubation with the eosinophils at different time points.

Furthermore, the biocompatibility of the persisted carbonaceous material was not assessed *in vitro* or *in vivo*.

Interestingly, Shvedova *et al.* found that pulmonary inflammatory responses induced by pharyngeal aspiration of oxidised SWNTs were more intense in myeloperoxidase enzyme knockout B6.129X1 mice compared to wild type C57BL/6 mice [76]. The authors attributed this observation to the SWNTs intracellular degradation by myeloperoxidase enzyme in the C57BL/6 mice. This was supported by the presence of a high proportion of SWNTs with shortened length and oxidative defects, in solubilised lung sample preparations, 28 days following exposure to the SWNTs as revealed from TEM and Raman spectroscopic analysis. However, the carbon material that withstood degradation appeared aggregated under TEM and the biocompatibility of the persisted material over the long term was not assessed. Moreover, biodegradation of the SWNTs following repeated administration or administration *via* other routes that are more frequently used in the biological applications of CNTs, e.g. intravenous or subcutaneous injection, were not investigated.

We have previously studied the degradation of amine-functionalised MWNTs in brain tissue following stereotactic brain injection [77]. TEM analysis of mouse brain tissue 2 days after the MWNTs administration (0.5 µg/mouse) showed the existence of intact cylindrical nanotubes and clustered non-tubular material in the microglia's cytoplasm, suggesting MWNTs biodegradation. This observation was further investigated *via* Raman spectroscopic analysis of brain tissues 2 and 14 days post MWNTs administration. Decreased intensity was observed in the MWNTs characteristic Raman spectra that could be attributed to biodegradation-induced defects, further suggesting degradation in brain tissue. However, dose-dependent degradation of the MWNTs and the possibility of complete degradation were not investigated.

Although the findings of these studies provide evidence for the tendency of CNTs towards intracellular biodegradation, the studies did not resolve the issue of the carbon-based material cellular persistence. Future studies have to investigate the cellular capacity to fully degrade the carbonaceous material and to find out how long would it take to completely degrade and eliminate a specified amount of CNTs. Furthermore, complete degradation of CNTs should not be the only concern of future studies, as the previous studies reported that CNTs biodegradation is achieved by the aid of intracellularly-generated reactive oxygen species. Thus, it is also important to carry out a long-term assessment of the cellular damage that may occur as a result of the increased oxidative stress following cellular uptake of CNTs.

Biodegradability and elimination can be considered as one of the major hurdles that could impede the clinical assessment of CNTs as delivery carriers to therapeutics. In a recent study published by Alidori *et al.*, SWNTs functionalised using 1,3-dipolar cycloaddition were intravenously administered to non-human primates (cynomolgus monkey), the injected SWNTs exhibited biodistribution profile and rapid renal elimination in a manner similar to that observed in mice [78]. Despite such encouraging results, clinical investigations of the CNTs proficiency as nanocarriers might not be possible until pre-clinical assessments of their biosafety, biodegradation and elimination are conclusive. In addition, clinical application of functionalised CNTs-based delivery systems could be also hindered by the necessity to produce them according to the current Good Manufacturing Practices. Hence, several obstacles will need to be overcome in order to meet these standards including uniformity of CNTs dimensions, functionalisation density and antigen loading.

3. Functionalised CNTs as cancer vaccine delivery system

Various types of particulate carriers have been utilised in vaccine delivery [79-83]. The efficacy of particulate vaccines has been assigned to a number of suggested mechanisms. Particulate delivery systems can accommodate multiple copies of the antigen and adjuvant. Hence, the uptake of particulate vaccine delivery systems by APCs could increase the antigen and adjuvant intracellular concentrations, thus the presented antigen density.

Potent anti-tumour immune response induction could be achieved *via* combinatorial therapeutic approaches consisting of tumour antigens and immune modulators capable of overcoming tumour-induced immune suppression [6, 84]. Examples of nanoscopic particulates previously employed to co-deliver tumour-derived antigen and adjuvant include liposomes (spherical vesicles consisting of lipid bilayers enclosing an aqueous core) [85], lipopolyplexes (lipid and DNA complex) [86], nanoparticles made up of emulsified poly(lactic-co-glycolic acid) (PLGA) [87], virus-like nanoparticles (self-assembled capsid protein lacking the viral nucleic acids) [88], albumin-based nanoparticles [89] or mesoporous silica nanoparticles (synthetic nanoparticles possessing porous structure) [90]. Examples of micro-sized carriers exploited in tumour antigen and adjuvant simultaneous delivery are polymeric systems consisting of PLGA [91] or diaminosulfide [92] polymers. Despite the differences in the composition and size of these nanoparticles or microparticles, they share the property of being spherical in shape. The efficacy of particulate delivery systems to deliver cancer vaccines comprised of antigen and adjuvant has been investigated in various studies [87, 89-102]. These studies have shown that nanoparticles (e.g. liposomes, PLGA nanoparticles or albumin nanoparticles) or microparticles (e.g. PLGA or diaminosulfide-based microparticles) co-incorporating antigen and adjuvant delayed the growth of cancer cells inoculated in

mice. This observation was attributed to the capacity of the nanoparticles or microparticles to augment the antigen-specific CD8⁺ T cell immune response elicited by the co-loaded antigen and adjuvant as demonstrated in these studies *in vitro* or *in vivo* [87, 89-102].

Inspired by the demonstrated potentials of the conventional spherical nano-systems as vaccine delivery vectors and by the CNTs capacity to enter the cells *via* different mechanisms, various studies have investigated the exploitation of CNTs as vaccine nanocarriers. As summarised in **Table 1**, these studies focused on functionalised CNTs using various approaches to deliver antigens expressed by cancer cells and/or adjuvants to APCs and tested the efficacy of the CNTs-delivered vaccines through the assessment of specific immune responses elicited *in vitro* and *in vivo*.

3.1 Functionalised CNTs as delivery vector for tumour-derived antigen

As a cancer vaccine delivery system, Sun *et al.* investigated the use of carboxylated MWNTs to deliver MCF7 breast cancer cells-derived tumour lysate protein (TumourP) to APCs, specifically the DCs [103]. Flow cytometry showed that the MWNTs improved the uptake of the covalently incorporated TumourP by DCs *in vitro*. Furthermore, DCs pre-treated with MWNT-TumourP were more efficient than DCs pre-treated with free TumourP in inducing lymphocyte-mediated cytotoxicity against the MCF7 cells *in vitro*. However, the capability of MWNT-TumourP in retarding the MCF7 breast cancer cells growth *in vivo* was not studied.

Meng *et al.* assessed the potentials of carboxylated MWNTs to augment the anti-tumour immune response elicited against covalently immobilised H22 liver cancer cell-derived tumour lysate protein (H22P) [104]. The H22 cells were subcutaneously inoculated into BALB/c mice and the mice were subcutaneously injected with

MWNT-H22P or free H22P starting 2 days post H22 cells inoculation. The injected treatments were further potentiated by the additional administration of inactivated H22 tumour cells as a tumour cell vaccine. The maximum anti-tumour response was observed in mice injected with MWNT-H22P. Lymphocytes isolated from MWNT-H22P injected mice showed higher cytotoxicity against the H22 cells *in vitro* than lymphocytes isolated from free H22P-injected mice. Additionally, some of the mice free of H22 tumour, as result of MWNT-H22P treatment, successfully inhibited H22 cell growth following re-administration. This was due to the induction of antigen-specific memory T cells, since challenging 'cured' mice with the unrelated breast cancer cell line EMT led to successful tumour growth. The authors suggested that the augmented antigen-specific immune response elicited by MWNT-H22P was due to increased uptake of MWNT-conjugated H22P by the APCs, however, the MWNTs ability to enhance antigen uptake by the APCs *in vivo* was not tested. In addition, to evaluate the therapeutic efficiency of MWNT-H22P, it would have been informative to treat H22-tumour bearing mice with the vaccine rather than to challenge mice with tumour cells post vaccination.

Table 1. Summary of the studies investigated the use of CNTs as vaccine delivery systems.

CNT	Antigen (conjugation)	Adjuvant (conjugation)	Significant outcome(s)	Refs
Carboxylated MWNT	MCF7 breast cancer cells-derived tumour lysate (covalent)	–	<ul style="list-style-type: none"> – Increased antigen uptake by DCs <i>in vitro</i>. – Improved the induction of tumour-specific T cell response by DCs <i>in vitro</i>. 	[103]
Carboxylated MWNT	H22 liver cancer cells-derived tumour lysate (covalent)	–	<ul style="list-style-type: none"> – Increased antigen-specific CTL response in mice. – Increased the cure rate of H22 tumours subcutaneously inoculated in mice. 	[104]
Amine-functionalised SWNT	Wilms' tumour protein-derived peptide (covalent)	–	<ul style="list-style-type: none"> – Increased the serum levels of peptide-specific IgG in mice. 	[105]
Polyethylene glycol-functionalised SWNT	–	CpG (non-covalent)	<ul style="list-style-type: none"> – Increased the CpG cellular uptake and CpG-mediated cytokine production by bone marrow-derived monocytes <i>in vitro</i>. – Delayed the growth of GL261 glioma intracranially implanted in mice. 	[106]
Carboxylated MWNTs	OVA or tumour-derived NY-ESO-1 (non-covalent)	CpG (non-covalent)	<ul style="list-style-type: none"> – Increased IFN-γ production by <i>ex vivo</i> stimulated splenocytes isolated from immunised mice spleen. – Increased the serum levels of antigen-specific IgG in mice. – Delayed the NY-ESO-1-B16F10 tumour growth in subcutaneous tumour model. 	[107]
Carboxylated and amine-functionalised MWNTs	OVA	CpG and α CD40 (non-covalent)	<ul style="list-style-type: none"> – Increased CD8⁺ T cells proliferation and IFN-γ production <i>in vitro</i>. – Increased antigen-specific CTL response in mice. – Delayed the OVA-expressing B16F10 tumour growth in subcutaneous tumour model and prolonged survival. – Delayed the OVA-expressing B16F10 tumour growth in lung pseudo-metastatic tumour model. 	[108]

In order to potentiate the immune response elicited by the poorly immunogenic Wilms' tumour protein (WT1) Villa *et al.* covalently conjugated WT1-derived peptide with amine-functionalised SWNTs [105]. The SWNTs were internalised by the DCs *in vitro*, as determined using live confocal imaging, with no effects exerted on cell viability. The free WT1 peptide or SWNT-WT1 peptide conjugate were mixed with an oil-based adjuvant and then subcutaneously administered to BALB/c mice. The highest levels of anti-WT1 peptide IgG were detected in the sera collected from mice vaccinated with SWNT-WT1 peptide. However, tumour therapy experiments using WT-1 expressing cells were not carried out.

3.2 Functionalised CNTs as delivery vector for adjuvants

Appropriate activation of APCs is crucial for unlocking their full T cell stimulatory capacity [109]. Innate activation of APCs is mediated by pattern recognition receptors (PRRs) such as Toll-like receptors (TLRs), C-type lectin receptors (CLRs) and NOD-like receptors (NLRs) [110, 111]. These receptors recognise ligands associated invading pathogens known as pathogen-associated molecular patterns (PAMP). Naturally occurring PAMPs or their synthetic analogues have been widely explored in vaccine formulations as adjuvants with the aim to promote immunity induction [111, 112]. In particular, synthetic TLR9 agonists in form of oligodeoxynucleotides (ODN) containing unmethylated deoxycytidine-deoxyguanosine dinucleotide (CpG) motifs have been included as adjuvant in many clinically investigated cancer vaccine formulations (**Fig. 3.**) [113, 114].

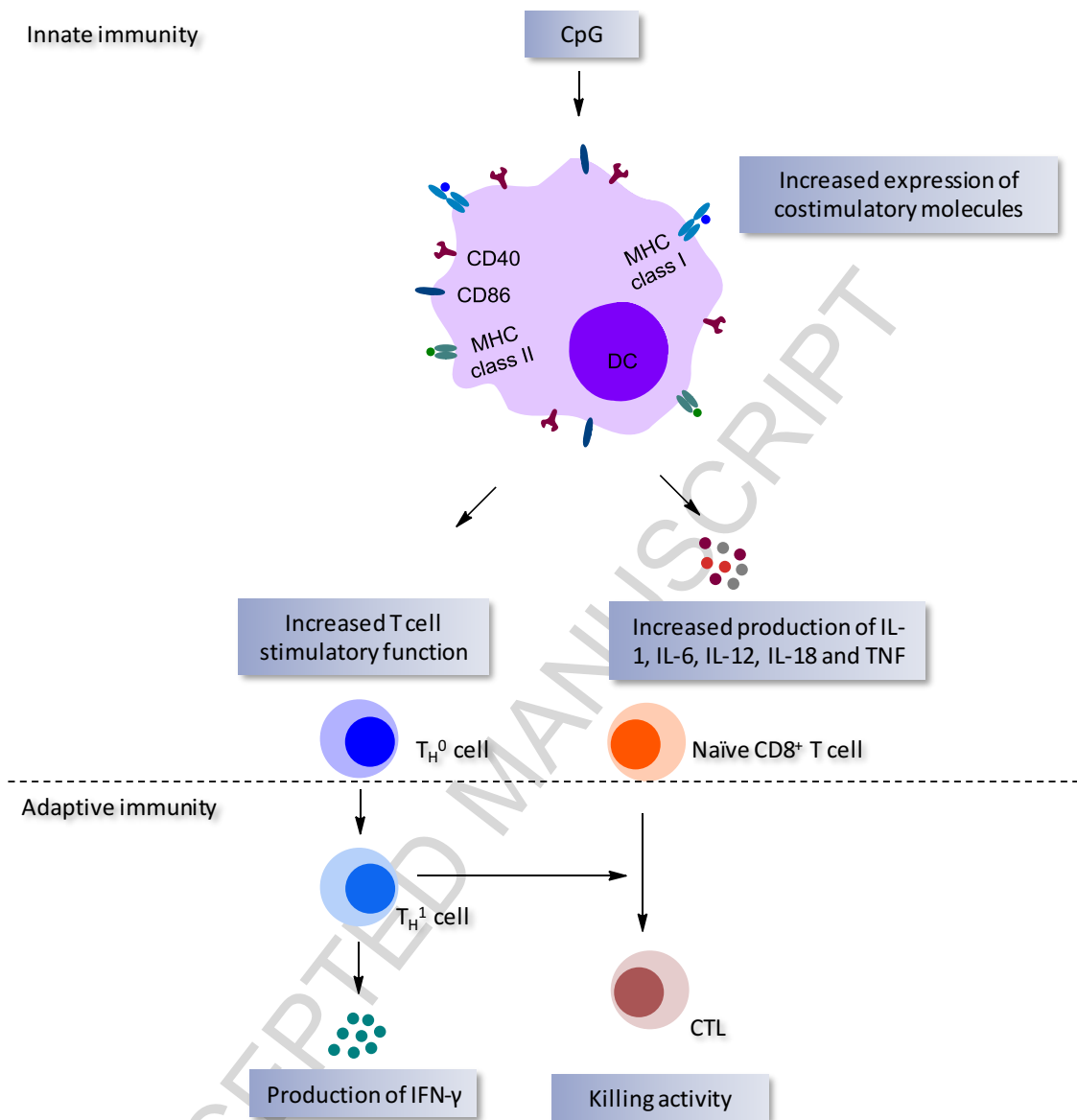


Fig. 3. Immune stimulatory activities of CpG. Stimulation of the DCs' TLR9 by CpG induces DC maturation by upregulating the MHC, CD40 and CD86 expression, and provokes the immune stimulatory cytokines production that ultimately determines the DC ability to induce CD4⁺ and CD8⁺ T cells priming. The figure is modified from ref [115].

The delivery of TLR9 agonist to APCs using CNTs has also been studied. Zhao *et al.* investigated whether SWNT-mediated delivery of CpG ODN could enhance the cellular uptake

and the immune stimulatory properties of the vaccine and promote potent anti-tumour immune response induction [106]. The authors showed that the uptake of fluorescently labelled CpG by bone marrow-derived monocytes *in vitro* was increased by its non-covalent conjugation to SWNTs. The SWNT-CpG conjugate also increased the CpG-induced production of interleukin (IL)-12 and tumour necrosis factor (TNF)- α by the monocytes *in vitro*. Moreover, intratumoural injection of the SWNT-CpG conjugate effectively delayed the growth of GL261 glioma cells intracranially-implanted in mice, which was not observed in mice injected with SWNT alone or free CpG. SWNT-CpG treatment failed to delay the growth of GL261 glioma cells intracranially implanted into mice depleted of CD8⁺ T cells or natural killer cells. This observation indicates the joint involvement of these cells in the induction of anti-tumour immune responses by SWNT-CpG conjugate.

Fan *et al.* have reported that intracranial administration of SWNT-CpG could also inhibit metastatic tumour growth in C57BL/6 mice [116]. This was demonstrated by showing that injection of SWNT-CpG was more efficient than free CpG in delaying the growth of subcutaneous and intracranial inoculated B16F10 melanoma cells. Furthermore, testing the therapeutic efficacy against a more challenging glioma model, Ouyang *et al.* have shown that SWNT-CpG along with the chemotherapeutic drug temozolomide, intratumorally administered, to mice bearing intracranial K-Luc glioma cells led to significantly prolonged survival in contrast to the injection of free CpG and temozolomide [117].

3.3 Functionalised CNTs as delivery vector for both tumour-derived antigen and adjuvants

In 2014 Faria *et al.* have reported the delivery of antigen and adjuvant to APCs using CNTs [107]. In this study the model antigen OVA and the TLR9 agonist CpG were both

non-covalently linked to carboxylated MWNTs. Mice immunised with MWNT-conjugated OVA and CpG showed higher sera levels of anti-OVA IgG and IFN γ production by *ex vivo* stimulated splenocytes than mice immunised with free OVA and CpG. The authors also tested the ability of the carboxylated MWNTs to co-deliver NY-ESO-1 (tumour antigen expressed in various human cancers) in combination with CpG to the APCs. The immune response induced *in vivo* by NY-ESO-1 and CpG was intensified following their non-covalent linkage to MWNTs. In addition, testing the ability to induce protective immunity, mice pre-injected with NY-ESO-1 and CpG-loaded MWNTs demonstrated a markedly retarded growth of NY-ESO-1 expressing B16F10 melanoma cells in subcutaneous tumour model. However, to assess the therapeutic efficacy of the designed MWNT-based vaccine, tumour-inoculated mice were vaccinated three days post tumour cell inoculation, when tumour growth was still undetectable. Therefore, it is unclear whether therapeutic vaccination with carboxylated MWNTs co-delivering antigen in combination to CpG ODN would lead to effective remission of a well establish tumour.

In a previous study, we compared the efficacy of MWNTs functionalised using 1,3-dipolar cycloaddition, oxidation or amide coupling reactions in delivering non-covalently immobilised OVA to APCs [118]. The MWNTs functionalised *via* amide coupling were more efficient in enhancing the OVA-specific immune response both *in vitro* and *in vivo*. In a follow-up study, the functionalised MWNT possessing surface chemistry that was found optimal for OVA delivery was then utilised for the co-delivery of OVA along with the adjuvants CpG and anti-CD40 antibody (α CD40) to the APCs in form of a (α CD40)MWNT(OVA-CpG) conjugate [108]. In addition to the immune stimulatory properties that can be acquired by the inclusion of α CD40 [84, 119, 120], we hypothesised that α CD40 contained in the aforementioned conjugate

will target it to the CD40 receptor on APCs, including cross-priming DC subsets, and thereby further enhance antigen cross-presentation to CD8⁺ T cells. Previous studies have demonstrated the ability α CD40-antigen conjugates to mediate uptake *via* CD40 receptor and enable translocation of the conjugate to the early endosomes [121-123]. It has been suggested that antigen translocation to early endosomes could support antigen escape into the cytosol and thereby promote antigen cross-presentation (**Fig. 4.**). It was observed that DCs treated with (α CD40)MWNT(OVA-CpG) *in vitro* possessed significantly lower surface expression of CD40 receptor compared to DCs treated with MWNT(OVA-CpG). This finding could support the hypothesis that CD40 receptor was internalised following its ligation with the α CD40 contained in (α CD40)MWNT(OVA-CpG) [121, 122]. However, to conclusively support this hypothesis it would be interesting to investigate the intracellular trafficking of the CD40 receptor following the incubation of DCs with (α CD40)MWNT(OVA-CpG). Antigen presentation to CD8⁺ T cells *in vitro* was more efficient when DCs were treated with (α CD40)MWNT(OVA-CpG) compared to treatment with free α CD40 in combination with MWNT(OVA-CpG). The (α CD40)MWNT(OVA-CpG) conjugate also amplified the OVA-specific CTL response *in vivo* at reduced OVA and CpG doses. Lastly, (α CD40)MWNT(OVA-CpG) demonstrated significant proficiency in delaying tumour growth in the subcutaneous and lung pseudo-metastatic B16F10-OVA tumour models. However, it is unclear whether antigen-specific memory was induced in this model since tumour-inoculated mice treated with (α CD40)MWNT(OVA-CpG) that remained tumour-free were not re-challenged with B16F10-OVA tumour cells.

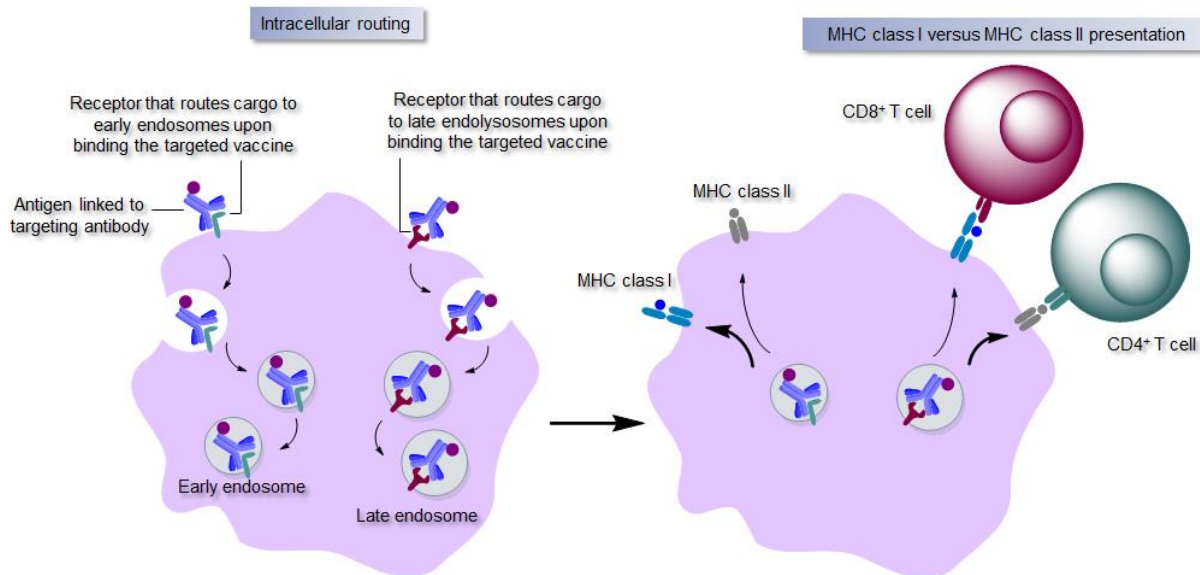


Fig. 4. Impact of intracellular routing of antibody-conjugated antigen on antigen presentation. Delivering antigens through a linked targeting antibody to a specific receptor expressed by APCs is followed by cellular internalisation of the formed cargo. Relying on the targeted receptor, the internalised cargo could be routed to a specific intracellular compartment. For instance, it has been found that mannose and CD40 receptors are intracellularly routed to the early endosomes, whereas CD205 receptor is routed to the late endosomes. In the late lysosome-containing endosomes, internalised antigen is subjected to rapid degradation by the lysosomal enzymes and presentation of the processed antigenic fragments *via* MHC class II molecules. In the early endosomes, the slow rate by which antigen is degraded could facilitate antigen escape from the endosomal to cytosolic compartments that allows proteasomal antigen degradation and, subsequently, presentation *via* the MHC class I molecules. The thicker arrow denotes that this route of presentation of processed antigen is more predominant than the other pathway. The figure has been re-drawn from ref [123].

4. Future perspective of CNTs as vaccine delivery systems

As with all cancer vaccines their success, or failure, is dependent on finding the right combination of antigen, adjuvant and delivery vehicle. The future of CNTs as a delivery platform/adjuvant is dependent on their relative potency compared to other modalities while the future of the field itself is inextricably linked to the identification of novel antigens or vaccine regimes. In the immediate future, CNTs need to be comprehensively assessed against other

vaccine formulations to establish where they belong on the ‘spectrum of adjuvanticity’. These studies are seldom performed (or at least seldom published) on novel adjuvant candidates due to the fear of coming up short against current formulations and thus losing funding/interest. However, it has made assessing whether an adjuvant is clinically viable over an alternate, near impossible.

Whether the outcome of these studies is positive or negative it should be noted CNTs have two distinct advantages over traditional adjuvants; the first is their mode of entry into cells. As previously reviewed CNTs have direct access to the cytosol through the proposed nano-needle mechanism. The implications of this in the context of vaccine adjuvants should not be understated. The ability to initiate a strong cytotoxic MHC class I restricted immune response to a soluble antigen has been seen as the Holy Grail for vaccine adjuvants. This is especially the case for cancer vaccines but also for vaccines against certain infectious agents [124, 125]. In addition, efficient delivery of payload to the cytosol may make other types of vaccines, specifically nucleic acid based vaccine including DNA and recently mRNA vaccines, more viable. The nano-needle mechanism is poorly understood with many groups reporting differing outcomes following administration of CNTs depending on a multitude of factors such as surface chemistry, size and aspect ratios as previously discussed. The first step in the rational design of a CNT based delivery vehicle should be the systematic evaluation of physical traits and surface modifications in order to improve cytosolic delivery. Our group has looked into some of the modifications of CNTs and has shown that relatively minor changes in structure can cause vast differences in immunogenicity though we have not been able to attribute this to improved cytosolic delivery [118]. Future work should continue and expand upon this.

The second key advantage of CNTs over traditional carriers is their large surface area. According to the current rationale for the use of CNTs, this enables the delivery of large amounts of antigen/adjuvant to APCs. Expanding on this theory, it has recently been shown that particulates can bind to antigen within the tumor environments following intratumoral inoculation [126]. For instance, Min *et al.* recently demonstrated that an injection of nanoparticle intratumorally, prior to radio ablation of the tumour, served to enhance the so called ‘abscopal effect’: the phenomenon whereby following the ablation of a tumour a systemic immune response is triggered against the released antigen/immune stimulants resulting in remission of distal tumours. One hypothesis is that the particles are retained within the tumour ‘mopping up’ antigen and immune active molecules. When the tumour is subsequently ablated the particles are released and are taken up by APCs in the tumour–draining lymph nodes [126]. The high surface area of the CNTs and the ability to form strong non–covalent associations with proteins specifically lends them to this purpose. Indeed it has been shown that GO formulated with CpG can be used for photothermal ablation, though the assessment of the abscopal effect was not measured, there is clearly an immune component [127]. There have been some studies assessing the ‘protein corona’ following administration of CNT intravenously, it would be interesting to perform these studies following intra–tumoral administration to determine how much tumour antigen can be absorbed directly from the tumour tissue in comparison to other carriers [128, 129]. Supporting this general hypothesis, it has been shown that CNTs, when formulated *in vitro* with tumour cell lysate, can serve to protect from tumour challenge [130].

Another intra–tumoral approach is the concept of ‘in situ’ vaccination. Here an agent is injected into the tumour leading to activation of the immune system to antigens present on the tumour/in the microenvironment. This relatively simple concept has led to miraculous results

preclinically, notably the use of CpG and anti-OX40 in combination is particularly potent [131]. It would be interesting to compare anti-OX40 to anti-CD40 antibody plus CpG in combination with CNT in a preclinical model to identify which approach is more efficient in eliciting anti-tumour immunity. It also would be interesting to compare the efficacy of synthetic CNT vaccines to biological vaccines such as plant virus-based vaccines. Filamentous and spherical plant viruses have been explored as in situ vaccines [132, 133]. In contrast to plant viruses, which are complex biological entities, CNTs may provide a better-controllable synthetic platform to explore the relationship between morphology and immunogenicity. In addition, CNT vaccines allow different combinations of TLR agonists to be used, and thereby enables the dissection of the requirements for optimal formulation.

In more general terms, it is likely that all future cancer vaccine candidates will be trialed in combination with a checkpoint inhibitor at some stage during their clinical assessment. To date, two checkpoint inhibitors have been clinically approved and these are targeted against inhibitory molecules PD-1 and CTLA4 and their purported mechanism has been reviewed elsewhere [15]. However, new classes of stimulatory checkpoint molecules including agonistic anti-OX40 and anti-4-1-BBL antibodies are showing efficacy in preclinical trials [120]. This has led to the concept of an 'immune switch'; this is a biomaterial containing both an immune stimulatory antibody as well as an anti-inhibitory antibody [134]. In this study the authors show that the immune switch is more potent than either of the two antibodies when administered in their soluble form. Again, due to their high surface area and ease at forming non-covalent attachments to proteins, CNTs could be the preferred vehicle for this purpose. Other than checkpoint blockade it could be argued that the generation of personalised vaccines becomes more feasible as DNA sequencing becomes faster and cheaper. The antigenic payload of

personalised vaccines will likely be peptides or nucleic acid due to ease of synthesis and quality control. However, both of these modalities are typically poorly immunogenic and require a delivery platform and adjuvant. In a proof of concept study the group of Kuai *et al.* sequenced murine tumours to detect mutations and these neo peptides were synthesised and loaded on to lipid nano discs [135]. The resulting particles were shown to protect against tumour challenge. In this approach it could be envisioned that the adjuvant/delivery platform will be prepared in a ready to use ‘off the shelf’ format and simply mixed with individual tumor neo antigens as determined by high throughput sequencing. CNTs represent a candidate platform for this approach as they can be synthesised in bulk and stored for long durations with little degradation, furthermore they can be made positively charged or amine reactive for binding of nucleic acid or peptide respectively as previously discussed.

5. Conclusions

The better understanding of tumour immunology has allowed development of cancer vaccines. However, tumour-induced immunosuppression has constituted a major hurdle to the capacity of these formulations to enhance the immune response. Attempts applied to augment anti-tumour immune response potency have included the delivery of the aforementioned vaccine formulations *via* nano-carriers. CNTs demonstrated characteristic cellular uptake properties that encouraged the exploitation of their nano-needle properties. Pre-clinical studies highlight that CNTs represent a competent cancer vaccine delivery system. Nevertheless, future studies are required to investigate the uniqueness that these CNTs possess as vaccine carrier over other vectors such as for example liposomes. In addition, despite the encouraging results reported in pre-clinical studies, clinical studies are hindered by the conflicting reports on CNTs biocompatibility and biodegradability. Finally, fortification of the vaccine formulations *via* the

inclusion of multiple adjuvants, APCs targeting ligands and combining this approach with a checkpoint inhibitor, could dramatically reduce the required CNTs doses and encourage their clinical assessment.

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	Antigen (conjugation)	Adjuvant (conjugation)	Significant outcome(s)
VNT	MCF7 breast cancer cells-derived tumour lysate (covalent)	–	<ul style="list-style-type: none"> – Increased antigen uptake by DCs <i>in vitro</i>. – Improved the induction of tumour-specific T cell response by DCs <i>in vivo</i>.
VNT	H22 liver cancer cells-derived tumour lysate (covalent)	–	<ul style="list-style-type: none"> – Increased antigen-specific CTL response in mice. – Increased the cure rate of H22 tumours subcutaneously inoculated in mice.
lised	Wilms' tumour protein-derived peptide (covalent)	–	<ul style="list-style-type: none"> – Increased the serum levels of peptide-specific IgG in mice.
lised	–	CpG (non-covalent)	<ul style="list-style-type: none"> – Increased the CpG cellular uptake and CpG-mediated cytokine production by bone marrow-derived monocytes <i>in vitro</i>. – Delayed the growth of GL261 glioma intracranially implanted in mice.
VNTs	OVA or tumour-derived NY-ESO-1 (non-covalent)	CpG (non-covalent)	<ul style="list-style-type: none"> – Increased IFN-γ production by <i>ex vivo</i> stimulated splenocytes isolated from immunised mice spleen. – Increased the serum levels of antigen-specific IgG in mice. – Delayed the NY-ESO-1-B16F10 tumour growth in subcutaneous tumour model.
nd lised	OVA	CpG and α CD40 (non-covalent)	<ul style="list-style-type: none"> – Increased CD8⁺ T cells proliferation and IFN-γ production <i>in vitro</i>. – Increased antigen-specific CTL response in mice. – Delayed the OVA-expressing B16F10 tumour growth in subcutaneous tumour model and prolonged survival. – Delayed the OVA-expressing B16F10 tumour growth in pseudo-metastatic tumour model.

Table 1. Summary of the studies investigated the use of CNTs as vaccine delivery systems.