Review

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Immunotherapy applications of carbon nanotubes: from design to safe applications

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Carbon nanotubes (CNTs) have the potential to overcome significant challenges related to vaccine development and immunotherapy. Central to these applications is an improved understanding of CNT interactions with the immune system. Unique properties such as high aspect ratio, flexible surface chemistry, and control over structure and morphology may allow for enhanced target specificity and transport of antigens across cell membranes. Although recent work has demonstrated the potential of CNTs to amplify the immune response as adjuvants, other results have also linked their proinflammatory properties to harmful health effects. Here, we review the recent advances of CNT-based immunological research, focusing on current understandings of therapeutic efficacy and mechanisms of immunotoxicology.

CNTs and immunotherapy: shaping a unique opportunity

As the field of nanomedicine evolves, the potential of CNTs (see Glossary) in medical applications has remained a controversial topic, fueled with concerns related to toxicity [1] and overhyped expectations [2]. Since their reintroduction in the early 1990s by Iijima [3], CNTs have been made more accessible for research through bulk production and advanced nanomanufacturing processes (for example, see: http://www-03.ibm.com/press/us/en/pressrelease/39250.wss). In the past decade, researchers have resiliently pushed for a better understanding of CNT properties [4], interactions with biological systems [5], and new methods involving safer material design [6]. Given the body's innate defense mechanisms, sensitivity in recognition of foreign agents, and subsequent regulation of the defense response, an important consideration is the interaction of CNTs with components of the immune system. The immunological relevance of CNTs has been motivated by the development of novel approaches for vaccine delivery with the design of 'nanosyringes' [7], engineering advanced cell culture platforms [8,9], but also significant concerns over their immunotoxicity *in vitro* and *in vivo* [1,10]. These approaches exploit the unique properties of CNTs such as shape and surface chemistry. CNTs are indeed known to exhibit a high aspect ratio defined by their longer tube dimension with respect to width during synthesis. Furthermore, CNTs have a strong graphitic character, and chemical strategies for the modification of the inert CNT

Glossary

Antigen-presenting cells (APCs): highly specialized cells that can process antigens and display their peptide fragments on the cell surface together with other, co-stimulatory, proteins required for activating naïve T cells. The main APCs for naïve T cells are dendritic cells, macrophages, and B cells.
Artificial antigen-presenting cells (aAPCs): cell-based and acellular technologies aimed at stimulating the therapeutic expansion and acquisition of T cells *ex vivo*.
Bundled CNTs: a set of CNTs wrapped or running close together due to hydrophobic interactions.
Carbon nanotubes (CNTs): CNTs are a class of carbon-based nanomaterial; they are graphene sheets rolled up into tubular form, thus, each layer consisting of carbon atoms forming hexagonal networks.
CpG oligodeoxynucleotide (ODN): single-stranded, unmethylated synthetic oligonucleotides containing a cytosine triphosphate deoxynucleotide followed by a guanine triphosphate deoxynucleotide (the 'p' refers to the phosphodiester link between consecutive nucleotides). CpG ODNs are recognized by TLR9 leading to strong immunostimulatory effects.
Debundled CNTs: a set of CNTs that are dispersed to their individual level.
Dendritic cells (DCs): a class of APCs derived from the bone marrow and found in most tissues, including lymphoid tissues. Functional cellular subsets include conventional, plasmasytoid, and follicular dendritic cells.
Graphene: a 2D crystalline allotrope of carbon.
Major histocompatibility complex (MHC) molecules: a set of membrane glycoproteins that includes MHC class I molecules, which present antigenic peptides generated in the cytosol to CD8 T cells, and MHC class II molecules, which present antigenic peptides generated in intracellular vesicles to CD4 T cells.
Multi-walled carbon nanotubes (MWNTs): a class of CNTs that are composed of several concentric sheets of graphene.
Natural killer (NK) cells: large granular, non-T, non-B lymphocytes, which kill virus-infected cells and some tumor cells.
Opsonization: alteration of the surface of a pathogen or other particle so that it can be ingested by phagocytes.
P stacking: quadropole interactions between delocalized electrons in p orbitals or between parallel aromatic p systems.
Pathogen-associated molecular patterns (PAMPs): molecules associated with groups of pathogens, which are recognized by cells of the innate immune system.
Single-walled carbon nanotubes (SWNTs): a class of CNTs that are composed of a unique layer of graphene.
SP2: the mixing of one s and two p atomic orbitals, which involves the promotion of one electron in the s orbital to one of the 2p atomic orbitals.
Toll-like receptor 9 (TLR9): a class of innate immune system receptors, expressed intracellularly within endosomal compartments, which recognizes unmethylated CpG sequences in DNA molecules [16].

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surface [11] represent a crucial step in enabling various biomedical applications. Overall, both the understanding and control of CNT physiochemical properties have allowed for new developments in targeted drug and genetic delivery [12], and in exploring the interaction of CNTs with cellular membranes [13] and physiological proteins [14,15]. Thus, the potential for CNTs to disturb the immune system – a tightly regulated network of tissues, cells, and molecules, and the basis for host defense against infection [16] – can have significant implications.

Immunotherapy refers to any approach aimed at manipulating or amplifying the immune system to better fight disease [17] (Box 1). Successful defense strategies have exploited antigenic targets to enhance therapeutic efficacy against chronic infectious diseases and cancer, and the blockade of regulatory mechanisms that can impede immunotherapeutic effects [17]. Vaccines exploit the ability of the immune system to recognize and react with foreign antigens [16]. The innate immune system forms the earliest barriers to infection that is not specific to any individual pathogen. An important component of the innate immune response is antigen uptake, processing, and presentation via specialized cells called antigen-presenting cells (APCs) [16]. These cells are phagocytic by nature, processing internalized antigen and displaying peptide fragments of these antigens on their surface in the context of major histocompatibility complexes (MHCs). Peptide–MHC complexes along with other co-stimulatory proteins and cytokines stimulate naïve T cells in the lymph nodes [16]. APCs also bridge the functional roles of innate and adaptive immunity. Thus, APCs, in addition to interacting with T cells, can activate innate natural killer (NK) cells and facilitate complement activation. Upon recognition of a pathogen, APCs, such as macrophages, dendritic cells (DCs), and B cells, are activated to initiate an adaptive antigen-specific immune response. Thus, successful vaccine strategies have exploited novel means to control APCs, whether it is the successful priming with a specific antigen to develop immunity, or tolerance induction by targeting of the specific antigen without inducing co-stimulation in APCs [18]. The profiling of cellular and molecular targets has indeed allowed researchers to improve the efficacy of therapeutic delivery systems by expanding the selection of vaccine vectors, adjuvants, and T cell signals, in addition to current advances in designing novel biomaterial-based platforms [19]. In this context, adapting CNT-based platforms for immunotherapy is beginning to gain significant momentum in the research community, with designs varying from nanosyringe-based vaccine vehicles and immunotherapeutic systems [20,21], to culture platforms focusing on the growth of cytotoxic immune cells [8].

In this report, we review relevant material and functional properties of CNTs for immunotherapeutic applications, including their interactions with molecules of the immune system. These properties result in unique opportunities for modulating key components of the immune response, including the activation of cells of the innate immune system such as APCs and complement proteins, as well as cells of the adaptive immune response. Furthermore, we discuss toxicity arising from unintended interaction of CNTs with various components of the immune system, and their relevant implications to safety by design. We conclude by providing some insights on the future of CNT-based immunological applications, and how toxicological studies will be key drivers enabling safe design of CNTs as a platform for immunotherapy.

Box 1. Forms of immunotherapy

- *Passive immunotherapy* involves the use of antibodies developed outside the body to protect or fight off a disease. These antibodies can be specific to antigens expressed by foreign pathogens, including tumors; yet, they do not trigger an active role from the host's immune system to fight the infection. This targeted, passive form of immunotherapy, which relies on the use of monoclonal antibodies (antibodies containing uniform variable regions) allow for various mechanisms of cytotoxicity against pathogens, including:
  - *Complement-dependent cytotoxicity*: activation of complement pathways via binding of antibody on target cell surface and resulting in the lysis of the antibody-coated cells.
  - *Antibody-dependent cell-mediated cytotoxicity:* activation of effector cells of the immune system via the binding of antibody on the target cell surface. Once the effector cell interacts with the fragment crystallizable region (Fc region), a signaling cascade is initiated within the cell resulting in the release of cytotoxic granules, thus triggering apoptosis of the antibody-targeted cell.
- *Active immunotherapy* involves the host immune system in taking an active role in fighting the disease. Active immunotherapy can be directly achieved *in vivo* with therapeutic vaccines, which aim to stimulate directly the adaptive arm of the immune system *in vivo*, or *ex vivo* through the adoptive transfer of effector lymphocytes.
  - *Vaccines:* current vaccine formulations include live-attenuated, killed-attenuated, or pathogen fragments, in addition to the potential use of an *adjuvant*, which boosts the immune response against the targeted antigen. Similarly, cancer vaccines use inactive tumor cells (whole tumor cell or cell extract, gene-modified cancer cells, or cancer cells fused to APCs) or genetic identifiers of cancer antigens (such as synthetic peptides, plasmids, recombinant viruses or bacteria).
  - *Adoptive transfer cellular therapy:* this method relies on cell populations that come directly from a patient after these cells have been naturally expanded and matured, or genetically modified *ex vivo*. In the case of cancer, specific population of T cells (tumor-infiltrating lymphocytes) can traffic to the tumor and trigger cancer cell death.

CNT structure and surface chemistry

CNTs are graphene sheets rolled up into tubular form, thus each layer consisting of carbon atoms forming hexagonal networks and bound to each other via strong sp2 bonds [22]. CNTs can be synthesized using various methods, from arc discharge to chemical vapor deposition and laser ablation [22]. Each of these techniques inevitably yields impurities that can be removed using various methods of chemical treatment. The presence of a unique layer of graphene characterizes single-walled carbon nanotubes (SWNTs), whereas several concentric sheets make up the structure of multi-walled carbon nanotubes (MWNTs). Dimensionally, these two classes of CNTs differ significantly by tube diameter and length. In general, SWNTs have a diameter ranging from 0.5 to 3.0 nm and length of 20–1000 nm, whereas the diameter of MWNTs ranges from 1.4 to 100 nm and the length from 1 to 50 μm. Carbon nanotubes are inherently insoluble in aqueous solutions and slightly soluble in organic solvents, and have a strong