Journal of Controlled Release 170 (2013) 242–251

Contents lists available at SciVerse ScienceDirect

Journal of Controlled Release

journal homepage: www.elsevier.com/locate/jconrel

Carbon nanotube-protein carriers enhance size-dependent self-adjuvant antibody response to haptens

Javier Parra [a], Antonio Abad-Somovilla [b], Josep V. Mercader [a], T. Andrew Taton [c], Antonio Abad-Fuentes [a,*]

[a] Department of Biotechnology, Institute of Agrochemistry and Food Technology (IATA), Spanish National Research Council (CSIC), Agustí Escardino 7, 46980 Paterna, Valencia, Spain
[b] Department of Organic Chemistry, Universitat de València, Doctor Moliner 50, 46100 Burjassot, Valencia, Spain
[c] Department of Chemistry, University of Minnesota, 207 Pleasant St. SE, Minneapolis 55455, USA

ARTICLE INFO

Article history:
Received 15 January 2013
Accepted 23 May 2013
Available online 2 June 2013

Keywords:
Antigen delivery
Antibodies
Azoxystrobin
Biomaterials
Carbon nanotubes
Immunization

ABSTRACT

Carbon nanotubes (CNTs) are nanomaterials with interesting emerging applications. Their properties make CNTs excellent candidates for use as new nanovehicles in drug delivery, immunization and diagnostics. In the current study, we assessed the immune-response-amplifying properties of CNTs to haptens by using azoxystrobin, the first developed strobilurin fungicide, as a model analyte. An azoxystrobin derivative bearing a carboxylated spacer arm (hapten AZc6) was covalently coupled to bovine serum albumin (BSA), and the resulting BSA–AZc6 conjugate was covalently linked to four functionalized CNTs of different shapes and sizes, varying in diameter and length. These four types of CNT-based constructs were obtained using efficient, fast, and easy functionalization procedures based on microwave-assisted chemistry. New Zealand rabbits and BALB/c mice were immunized with BSA–AZc6 alone and with the four CNT–BSA–AZc6 constructs, both with and without Freund's adjuvant. The IgG-type antibody responses were assessed in terms of the titer and affinity, paying special attention to the relationship between the immune response and the size and shape of the employed CNTs. Immunization with CNT–BSA–AZc6 resulted in enhanced titers and excellent affinities for azoxystrobin. More important, remarkable IgG responses were obtained even in the absence of an adjuvant, thus proving the self-adjuvanting capability of CNTs. Immunogens were able to produce strong anti-azoxystrobin immune responses in rabbits even when administered at a BSA–AZc6 conjugate dose as low as 0.05 μg. The short and thick CNT–BSA–AZc6 construct produced the best antibody response under all tested conditions.

1. Introduction

Enhancement of the immune response against antigens is an area of great interest because of its impact on antibody production. One of the key steps in the immunization process is the phagocytosis of the antigen by professional antigen-presenting cells (APCs), such as macrophages, dendritic cells, and certain B cells [1,2]. Once internalized by APCs, the antigen is processed into short peptide fragments. The presentation of these peptides by major histocompatibility complex (MHC) class II molecules can activate helper T cell responses that ultimately lead to the proliferation of plasma and memory B cells and to the production of high-affinity antibodies [3–6].

The efficiency of the phagocytosis process mainly depends on the size and surface properties of the antigen. Large and insoluble particulate antigens are phagocytosed very efficiently and produce strong responses by themselves [7–9]. This is the case for microorganisms such as bacteria and viruses [10,11]. Conversely, small and soluble antigens, such as proteins and peptides, are poorly internalized and thus weakly immunogenic; therefore, an adjuvant is needed to intensify the immune response [12,13]. Triggering an efficient immune response to low-molecular-weight compounds (haptens) is an even more challenging task because they are smaller and lack a peptide structure, so they cannot be presented by MHC II molecules unless they are conjugated to proteins prior to immunization [14,15].

One of the emerging strategies for increasing the efficiency of the phagocytosis step, thereby enhancing the antibody response, is the use of nanomaterials that can increase the size of the antigen and modify its surface properties. Micro/nanoparticles (MPs/NPs) have been shown to be well-suited for increasing the immune response against protein and peptide antigens because the dimensions of particulate systems are comparable to those of microorganisms. Therefore, they can be better engulfed by APCs and the antigens can be delivered more efficiently [16]. There are basically two possible ways to take advantage of MPs/NPs for immunization purposes. One way consists of encapsulating the antigen inside liposomes [17,18] or inside organic biodegradable polymers, such as poly(lactic acid) (PLA), poly(lactic-*co*-glycolic acid) (PLGA) [19–24], disulfide cross-linked polyacrylates [25], or polysaccharides, such as pullulan [26] and chitosan [27–29]. This way, the particles can easily reach cytosol, where they are degraded, thus releasing the antigen.

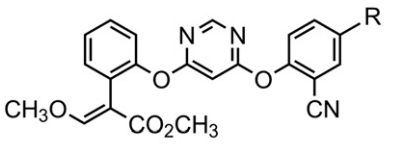
* Corresponding author. Department of Biotechnology, Institute of Agrochemistry and Food Technology (IATA), Spanish National Research Council (CSIC), Agustí Escardino 7, 46980 Paterna, Valencia, Spain. Tel.: +34 963 900 022; fax: +34 963 636 301.
E-mail address: aabad@iata.csic.es (A. Abad-Fuentes).

0168-3659/$ – see front matter © 2013 Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.jconrel.2013.05.019

The other approach consists of attaching the antigen (covalently or not) to the functionalized surface of biocompatible and non-biodegradable particulate materials, such as lecithin NPs [30], gold NPs [31–33], quantum dots [34], or MPs/NPs based on diverse organic polymers like polystyrene [35,36], polypropylene sulfide [37], or polyacrylate [38].

Carbon nanotubes (CNTs) are nanomaterials of tubular shape with promising applications in biological and medicinal chemistry [39–43]. They are capable of crossing membranes to penetrate into different types of cells without causing cell death, so both the cell functionality and the activity of the introduced molecules are preserved [44–46]. Based on this finding, CNTs have been successfully employed as nanovehicles for drug delivery [43,47,48] and for the transport of biomolecules, such as proteins [49], peptides [50], and DNA [42,51,52], into cells. Additionally, based on their structural and chemical properties, CNTs can be envisioned as excellent carrier candidates in immunization protocols. They are very hydrophobic and insoluble because of their carbon skeleton structure, and they have a size on the micrometer/nanometer scale, mimicking the properties and size of bacteria. If an antigen is linked to a CNT, the resulting construct could be more easily phagocytosed and rapidly introduced into the immune system, thus improving the antibody response. Pantarotto et al. [53,54] demonstrated the potential of using CNTs for animal immunization. In their seminal work, a peptide from the foot-and-mouth disease virus (FMDV) was covalently coupled to amino-derivatized single-walled carbon nanotubes (SWNTs), and this conjugate was administered to mice. Their results showed that this derivative, together with ovalbumin and an adjuvant, elicited high virus-neutralizing antibody responses in comparison with the non-conjugated peptide. Since this work, the advantages of immunization with proteins and peptides using CNTs have occasionally been confirmed [55–58], but, to the best of our knowledge, no evidence of an enhancement in the immune response and antibody production against small molecules using CNTs as vehicles has been reported. The production of antibodies displaying high-affinity and specificity to haptens is an important biotechnological research field because tailor-made binders can be used to develop immunoassays and biosensors for targets such as medicinal drugs, pesticides, drugs of abuse, environmental contaminants, food additives, hormones, and toxins.

In the present work, we explore for the first time the potential of CNTs as self-adjuvanting complexes in order to amplify the antibody response to haptens. Azoxystrobin, a molecule with three aromatic rings interconnected by ether linkages (Chart 1), was selected as the model hapten for this study. Azoxystrobin was the first discovered and patented antifungal of the strobilurin agrochemical family [59]. It is currently the world's best-selling proprietary fungicide with global annual sales over $1 billion [60], which makes the development of rapid immunochemical methods for this target a desirable goal. To evaluate the efficiency of CNTs as vehicles for immunization, rabbits and mice were injected with covalent CNT–BSA–hapten constructs. Special attention was paid to the relationship between the antibody response and the CNT size and shape. Animal immunization was carried out with functionalized single- and multi-walled nanotubes (SWNTs and MWNTs, respectively) that were either shortened or not by an acid oxidative treatment, so four types of CNTs differing in length and diameter were obtained and evaluated. The preparation of this group of functionalized CNTs was performed using fast and easy procedures based on optimized microwave-assisted chemical methods. Finally, the ability of CNTs to efficiently trigger an immune response by themselves, without the need for conventional adjuvants, was also investigated.



azoxystrobin: R = H

hapten (AZc6): R = $(CH_2)_5COOH$

Chart 1. Structure of azoxystrobin and of the AZc6 hapten.

2. Materials and methods

2.1. Chemicals and instrumentation

Pristine SWNTs (90 wt.%, 1–2 nm in diameter, 5–30 μm in length) and MWNTs (95 wt.%, 50–80 nm in diameter, 10–20 μm in length) were purchased from Cheap Tubes Inc. (Brattleboro, VT, USA) and used as received. Solvents and reagents for CNT functionalization and for immunogen preparation were obtained from Sigma-Aldrich (St. Louis, MO, USA) and used without further purification. Succinic acid peroxide was obtained from the H_2O_2 treatment of succinic acid anhydride, as previously described [61]. The synthesis of hapten AZc6, as well as the preparation and purification of the immunizing BSA–AZc6 conjugate (**5**) and the coating conjugate OVA–AZc6 for the indirect competitive ELISA, has been previously described [62].

Microwave reactions were performed in a CEM Discover S-Class reactor (Matthews, NC, USA) equipped with an infrared pyrometer for temperature control, a pressure control system, magnetic stirring, and a simultaneous air-cooling option. Open vessel reactions were conducted using conventional glassware, whereas pressurized reactions were performed in CEM supplied microwave quartz special vessels. Nylon membranes with a pore size of 0.45 μm were purchased from GE Power & Water (Trevose, PA, USA) for vacuum filtration of CNT samples. The reaction progress was observed by measuring the zeta potential of the samples in deionized water using a Malvern Zetasizer Nano ZS apparatus (Worcestershire, UK). TEM images were obtained using a JEOL 100 kV JEM-1010 microscope (Tokyo, Japan) equipped with a MegaView III digital camera and with the *Analysis* image acquisition software.

Azoxystrobin was kindly provided by Syngenta (Basel, Switzerland). Concentrated stock solutions were prepared in anhydrous DMF and stored at −20 °C. Polyclonal goat anti-rabbit IgG–peroxidase antibody (GAR–HRP) was obtained from BioRad (Madrid, Spain). Polyclonal rabbit anti-mouse IgG–peroxidase antibody (RAM–HRP) was obtained from Dako (Glostrup, Denmark). Polyclonal goat anti-mouse IgG–gold antibody (5 nm colloidal gold), *o*-phenylenediamine, complete and incomplete Freund's adjuvants, Tween 20 and other reagents for immunoassays were purchased from Sigma-Aldrich (Madrid, Spain). The preparation of the mouse IgG anti-AZc6 mAb was previously described [63]. Costar 96-well, flat-bottom, high-binding, polystyrene ELISA plates were obtained from Corning (Corning, NY, USA). The microplates were washed using an ELx405 microplate washer from BioTek Instruments (Winooski, VT, USA), and the ELISA absorbances were read using a PowerWave HT reader, also from BioTek Instruments.

2.2. Oxidative fragmentation procedure

100 mg of SWNTs or MWNTs was suspended in 25 mL of a 70% HNO_3 solution in a microwave quartz vessel and sonicated for 15 min. The vessel was closed, and the mixture was irradiated for 30 min at 190 °C and at 250 W under a pressure of 19 bar with magnetic stirring and simultaneous air-cooling. Next, the cooled reaction mixture was diluted in 250 mL of deionized water and filtered through a 0.45 μm nylon membrane. The resulting black solid was collected, resuspended in 100 mL of water, sonicated, filtered again, and washed with water. Finally, the air-dried solids were suspended in deionized water at a concentration of 1 mg/mL and sonicated for 5 min to obtain stable solutions of **1a** and **2a** with zeta potential values of −35 and −50 mV, which were stored at 4 °C.