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Physicochemical and biological characterization of single-walled and double-walled carbon nanotubes in biological media

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HIGHLIGHTS

- Investigation of current dispersal method for nanotoxicology.
- BSA and BALF were used to investigate protein and CNT interaction.
- Alteration in physicochemistry of CNT was induced by BSA.
- Protein binding to CNT could result in misinterpretation of *in vitro* results.
- Protein-to-CNT interactions were associated with the coagulation pathways.

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ABSTRACT

To study the toxicity of nanoparticles under relevant conditions, it is important to reproducibly disperse nanoparticles in biological media in *in vitro* and *in vivo* studies. Here, single-walled nanotubes (SWNTs) and double-walled nanotubes (DWNTs) were physicochemically and biologically characterized when dispersed in phosphate-buffered saline (PBS) and bovine serum albumin (BSA). BSA-SWNT/DWNT interaction resulted in a reduction of aggregation and an increase in particle stabilization. Based on the protein sequence coverage and protein binding results, DWNTs exhibited higher protein binding than SWNTs. SWNT and DWNT suspensions in the presence of BSA increased interleukin-6 (IL-6) levels and reduced tumor necrosis factor-alpha (TNF-α) levels in A549 cells as compared to corresponding samples in the absence of BSA. We next determined the effects of SWNTs and DWNTs on pulmonary protein modification using bronchoalveolar lavage fluid (BALF) as a surrogate collected form BALB/c mice. The BALF proteins bound to SWNTs (13 proteins) and DWNTs (11 proteins), suggesting that these proteins were

Abbreviations: BSA, bovine serum albumin; BALF, bronchoalveolar lavage fluid; DWNTs, double-walled nanotubes; DLS, dynamic light scattering; EDX, energy-dispersive X-ray; HRTEM, high-resolution transmission electron microscopy; IFN-γ, interferon-gamma; IL-6, interleukin-6; LC–MS, liquid chromatography–mass spectrometry; NTs, nanotubes; PANTHER, Protein ANalysis THrough Evolutionary Relationships; PBS, phosphate-buffered saline; SEM, scanning electron microscope; SWNTs, single-walled nanotubes; SDS-PAGE, sodium dodecyl sulphate-polyacrylamide gel electrophoresis; TFA, trifluoroacetic acid; TNF-α, tumor necrosis factor-alpha.

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associated with blood coagulation pathways. Lastly, we demonstrated the importance of physicochemical and biological alterations of SWNTs and DWNTs when dispersed in biological media, since protein binding may result in the misinterpretation of *in vitro* results and the activation of protein-regulated biological responses.

1. Introduction

Single-walled nanotubes (SWNTs) and double-walled nanotubes (DWNTs) are hollow tubes comprising a single wall or two walls, respectively, of crystalline graphene. The possible biomedical applications of NTs have attracted a great deal of interest as novel drug delivery systems [1], imaging contrast agents [2] and detection devices for tumor cells in the blood [3]. Increasing evidence suggests that NTs induce oxidative stress [4], inflammation [5] and immunotoxicity [6]. Xu et al. [7] also demonstrated that multi-walled nanotubes translocated into the pleural cavity and induced visceral mesothelial proliferation in rats. Therefore, the potential adverse human health impact of the biomedical use of NTs must be thoroughly evaluated prior to its widespread application.

Aggregation and the more dynamic process of agglomeration are important issues when examining nanoparticle toxicity. Aggregation indicates strongly bonded or fused nanoparticles, whereas agglomeration indicates more weakly bonded nanoparticles [8]. Agglomeration and aggregation are both associated with nanoparticle toxicity due to their regulation of cellular uptake mechanisms [9]. Therefore, increasing evidence has suggested that physicochemical characterization is required to explore the toxicity of nanoparticles [10]. Previous toxicological studies have physically investigated nanoparticle agglomeration in solutions in the presence and absence of protein addition. For example, Zhang and colleagues [11] illustrated that serum-containing medium thoroughly disaggregated titanium dioxide nanoparticles after 120 h. Vippola et al. [12] investigated the addition of bovine serum albumin (BSA) to cell culture media, which reduced agglomerates and increased stability. Surfactants are commonly used to disperse nanoparticles for toxicological investigations [11–13]. For sample, BSA, a type of surfactant, is a globular serum protein that is commonly used in biochemical studies. BSA consists of 583 amino acid residues and its molecular weight is 66,463 Da. The structure of BSA is composed of 67% alpha helices and 17 disulphide bridges, thereby increasing its stability [14]. The advantages of serum-containing media, such as media containing BSA, are that the addition of BSA can disaggregate and stabilize the size of nanoparticles, allowing for a better understanding of nanoparticle toxicity under consistent physicochemical conditions. However, the interactions between proteins and NTs and the consequent biological effects remain unclear.

Nanoparticle surface chemistry is a considerable factor in controlling the binding of proteins to specific ligands, resulting in inflammation and immunity [6,15]. Furthermore, protein modification may regulate cell morphology, cell communication and enzymatic processes, leading to abnormal cellular homeostasis. However, the interaction of NTs with proteins and the consequent responses of biological systems are less well understood and are important for the evaluation of nanoparticle toxicity. To perform this investigation, we physicochemically characterized the interaction of SWNTs and DWNTs with BSA. Next, we examined BSA modification by SWNTs and DWNTs [using liquid chromatography–mass spectrometry (LC–MS)] and consequent biological responses [indicated by interleukin-6 (IL-6), interferon-gamma (IFN-γ) and tumor necrosis factor-alpha (TNF-α) levels] *in vitro* in the presence and absence of BSA. Proteins in murine bronchoalveolar lavage fluid (BALF) were allowed to interact with SWNTs and DWNTs in order to examine the bindability of the protein–particle conjugates. Lastly, SWNT- and DWNT-specific proteins were identified, and the biological processes, molecular functions and pathways affected by the protein–particle conjugates were analyzed.

2. Materials and methods

2.1. Sample preparation

Two near-pure, manufactured, chemical-less carbon NTs were used: SWNTs and DWNTs. The SWNTs and DWNTs were purchased from Nanostructured & Amorphous Materials Inc. (Houston, USA). The detailed parameters were as follows: Outer diameters of 1–2 nm for SWNTs and 5 nm for DWNTs, lengths of 1–3 μm for SWNTs and 5–15 μm for DWNTs, purities >95% and special surface areas of 360–400 m^2/g for SWNTs and 400 m^2/g for DWNTs. The preparation of SWNTs or DWNTs in protein solution was based on protocols outlined in previous studies [16–18]. Briefly, filtered BSA at 1 mg/ml was prepared in sterile phosphate-buffered saline (PBS). Following incubation at 37 °C for 2 h under constant shaking at 500 rpm, SWNTs or DWNTs were suspended and sonicated in two solutions (BSA and BSA-free) at final concentrations of 0, 50, 150 and 1000 μg/ml. In the present study, three SWNT and DWNT fractions were used, as follows: (1) supernatant (unbound protein), (2) pelleted particles (protein bound to particles) and (3) suspension. The supernatant fraction (1) was defined as the filtered supernatant collected from the centrifuged particle–protein mixture. The pelleted fraction (2) was denoted as the pelleted particles obtained after centrifugation without the supernatant fraction. The suspension fraction (3) was described as the well-mixing particle–protein samples. To obtain the supernatant and pelleted fractions, the samples (0, 50, 150 and 1000 μg/ml of SWNTs or DWNTs) were then separated into two fractions using centrifugation (3500 rpm, or approximately 2100 × *g*) and filtration following a PBS wash. In the present study, the three SWNT and DWNT fractions were used in different bioreactive investigations. First, protein modification in the SWNT and DWNT supernatant fractions and the corresponding pelleted fractions was determined. Second, inflammation and immune responses that were caused by the SWNT and DWNT suspension fractions and their corresponding supernatant fractions were investigated *in vitro*. NT-free samples prepared in BSA or a BSA-free solution served as negative controls with the corresponding treatment. All chemicals that were used in the present study were of reagent grade (Sigma–Aldrich, UK), unless otherwise stated.

2.2. NT characterization

The BSA-containing and BSA-free pelleted fractions were lyophilized for analysis using a scanning electron microscope (SEM). The preparation of SEM samples has been previously reported [19]. Briefly, the samples were fixed onto 13 mm aluminum SEM stubs after platinum coating (at an average thickness of 10 nm). An Inspect™ SEM (FEI, USA) was used to investigate the morphologies of samples at a voltage of 15 kV and a spot size of 2.5. The *energy-dispersive X*-ray (EDX) Genesis Microanalysis