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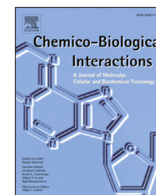

Acute toxicity of functionalized single wall carbon nanotubes: A biochemical, histopathologic and proteomics approach

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A B S T R A C T

Recently carbon nanotubes (CNTs) showed promising potentials in different biomedical applications but their safe use in humans and probable toxicities are still challenging. The aim of this study was to determine the acute toxicity of functionalized single walled carbon nanotubes (SWCNTs). In this project, PEGylated and Tween functionalized SWCNTs were prepared. BALB/c mice were randomly divided into nine groups, including PEGylated SWCNTs (75,150µg/mouse) and PEG, Tween80 suspended SWCNTs, Tween 80 and a control group (intact mice). One or 7 days after intravenous injection, the mice were killed and serum and livers were collected. The oxidative stress markers, biochemical and histopathological changes were studied. Subsequently, proteomics approach was used to investigate the alterations of protein expression profiles in the liver.

Results showed that there were not any significant differences in malondealdehyde (MDA), glutathione (GSH) levels and biochemical enzymes (ALT and AST) between groups, while the histopathological observations of livers showed some injuries. The results of proteomics analysis revealed indolethylamine N-Methyltransferase (INMT), glycine N-Methyltransferase (GNMT), selenium binding protein (Selenbp), thioredoxin peroxidase (TPx), TNF receptor associated protein 1(Trap1), peroxiredoxin-6 (Prdx6), electron transport flavoprotein (Etf-α), regucalcin (Rgn) and ATP5b proteins were differentially expressed in functionalized SWCNTs groups. Western blot analyses confirmed that the changes in Prdx6 were consistent with 2-DE gel analysis. In summary, acute toxicological study on two functionalized SWCNTs did not show any significant toxicity at selected doses. Proteomics analysis also showed that following exposure to functionalized SWCNTs, the expression of some proteins with antioxidant activity and detoxifying properties were increased in liver tissue.

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1. Introduction

Carbon nanotubes (CNTs) are a widely used family of nanomaterials. According to the number of graphene layers, these hollow CNTs are divided to SWCNTs (Single Wall Carbon Nanotube) and MWCNT (Multi Wall Carbon Nanotube). SWCNTs have remarkable thermal, electronic and optical properties [1,2]. Besides

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broad range industrial applications, SWCNs have many potential medical applications including drug and gene delivery, tissue regeneration and artificial implants [3–5].

As pristine carbon nanotubes are intrinsically water insoluble and have a hydrophobic nature, functionalization and surface modification are required to make them compatible with biomedical applications [6]. There are two approaches for SWCNTs functionalization, covalent and noncovalent attachment (physioadsorption) [7]. By functionalization, CNTs become hydrophilic and ready for linking with drugs or biomolecules (genes, DNA, proteins, enzymes, biosensors, etc.) to deliver them into the target cells or organs. Many reports have shown that properly functionalized CNTs are valuable nanocarriers for efficient and safe delivery of biomolecules [8,9]. Previously we showed that proper functionalized CNTs can be used as efficient gene delivery vectors with low cytotoxicity in vitro and in vivo [10–16]. For biomedical applications, various functionalization strategies can be used and each method has its own advantages. Non-covalent functionalization of CNTs with amphiphilic molecules is of great importance, because in contrast to covalent functionalization, the graphene-network of sheets is not disturbed, and their extraordinary physical properties remain intact [17]. In contrast, when a reliable strong link between nanotube and biomolecule is necessary, covalent functionalization is more valuable. Herein we decided to use two simple and reliable functionalization methods for SWCNTs. Therefore for covalent functionalization of SWCNTs, Polyethylene glycol (PEG) as a hydrophilic polymer with high biocompatibility and dispensability was used [18,19]. On the other hand, Tween 80 as a safe biocompatible and commercially inexpensive surfactant was used for noncovalent functionalization of nanotubes.

Many factors including route of administration, type of functionalization and the nature of tissues strongly affects the toxicity of SWCNTs. It has been shown that a proper functionalization can reduce the toxicity of pristine carbon nanotubes [20].

Although several studies were designed to evaluate the toxicity of pristine or functionalized SWCNTs, few data on acute toxicity of intravenously injected SWCNTs have been reported [6,7,9,17–19].

In this study we applied proteomics analysis, oxidative stress and histological examination to assess the potential changes in response to acute high dose of functionalized SWCNTs in order to provide a more comprehensive understanding of the safety of these nanomaterials in biomedical application.

2. Materials and methods

2.1. Materials

A preparation of purified SWNTs, produced by the HiPco method (0.8–1.2 nm individual diameter and 100–1000 nm length according to manufacturer specifications) was purchased from Unidym Inc. (Sunnyvale, USA). IPG strips (pH 3–10, 17 cm), Bio-Lyte (pH 3–10), the fluorescent SYPRO R Ruby protein gel stain, and protein assay kit were purchased from BioRad (USA). MDA, Acrylmide, SDS, urea, thiourea, Tris-HCl, and glycine were provided by Merck (Germany). Mineral oil, CHAPS, DTT, and iodoac-Glutathione, DTNB, Protease Inhibitor Cocktail, PMSF were purchased from Sigma-Aldrich and ultrapure agarose was obtained from Invitrogen (USA). Molecular weight marker was obtained from Fermentase (USA).

2.2. Fuctionalization, purification and characterization of SWCNTs

2.2.1. Noncovalent functionalization of CNTs

1 mg CNTs and 5 mg Tween80 were dispersed in 5 cc deionized water. Then, mixture was sonicated for 1 h in 20–40 °C. The resulting supernatant was centrifuged at 3000 g for 5 min to separate pellets. Then, the supernatant was filtered through Amicon filters with 100 kDa and washed 5 times. Finally, 1 mg/mL Tween functionalized CNTs solution was prepared and stored at 2–5 °C.

2.2.2. Covalent functionalization of CNTs

In order to introduce carboxylic acid functional groups onto the surface of SWNTs, modified oxidation procedure was used [20]. Briefly, 30 mg SWNT was dispersed in 100 ml HNO_3 2.5 M and sonicated for 2 min. The mixture was refluxed for 48 h and then sonicated for 30 min. The mixture was refluxed for another 24 h. The reaction mixture was filtered using PTFE membrane (200 nm) and the filtrate was washed with distilled water several times. The product (SWNT_COOH) was dried at 60 °C overnight. The second step was synthesis of PEG-functionalized SWNT (SWNT-PEG) as follow, 30 mg of the final product of the oxidation procedure (SWNT-COOH) was dispersed in 5 ml distilled water and sonicated for 2 min. Then 191.7 mg EDC and 115.09 mg NHS was added and the mixture was stirred for 45 min at room temperature. Then, 200 mg NH2-PEG-COOH (MW 3400) was added and the reaction was completed after 3 days. The reaction mixture was filtered with PTFE membrane and the supernatant was dialyzed (KD cutoff) for 3 day against 2 L of distilled water and then lyophilized.

2.3. Characterization of functionalized carbon nanotubes

Size and zeta potentials characterizations were carried out by using Zetasizer (Nano-ZS, Malvern Instruments, UK) [20]. Also Thermogravimetric analysis (TGA) was done with the TGA 50 instrument (Shimadzu, Japan) by heating the sample at a rate of 10 °C min^{-1} to 800 °C in air.

2.4. Animals

All animal experiments were performed in compliance with the Institutional Ethical Committee and Research Advisory committee of Mashhad University of Medical Sciences based on the national guidelines from Ministry of health and medicinal education of Iran.

Four weeks old BALB/c mice were obtained from Pasteur Institute (Tehran, Iran), and weighted 25–27 g at the time of experiment. They were acclimatized before performing the study with free access to food and water.

2.5. Experimental design

There were four treatment groups, PEG, PEGylated SWCNTs, Tween 80, Tween 80 suspended SWCNTs and a control group consisted of five untreated mice. Each treatment group was also composed of five animals. Two doses of 75 or 150 μg per mouse were administrated to functionalized SWCNT groups (Tween 80 suspended and PEGylated SWCNTs) and 200 μg of PEG or Tween80 per mouse were administrated to PEG and Tween 80 groups. These dosages were chosen according to the previous study with some modification after a pilot study [3].

In order to evaluate acute toxicity, mice were sacrificed one or seven days following the exposure. Serum and Liver tissues were separated. Tissues were stored at −80 °C for further experiments.

2.6. Measuring the MDA in the liver tissue

In order to measure MDA, as a marker of oxidative stress, the liver tissues, were homogenized in 1.15% KCl for 2 min at 4 °C (POLYTRON PT 10–35, Kinematic, Switzerland) to provide a 10% homogenate. MDA levels were determined as reported by

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