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Structural profiling and biological performance of phospholipid–hyaluronan functionalized single-walled carbon nanotubes

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ABSTRACT

In spite of significant insolubility and toxicity, carbon nanotubes (CNTs) erupt into the biomedical research, and create an increasing interest in the field of nanomedicine. Single-walled CNTs (SWCNTs) are highly hydrophobic and have been shown to be toxic while systemically administrated. Thus, SWCNTs have to be functionalized to render water solubility and biocompatibility. Herein, we introduce a method for functionalizing SWCNT using phospholipids (PL) conjugated to hyaluronan (HA), a hydrophilic glycosaminoglycan, with known receptors on many types of cancer and immune cells. This functionalization allowed for CNT solubilization, endowed the particles with stealth properties evading the immune system, and reduced immune and mitochondrial toxicity both *in vitro* and *in vivo*. The CNT–PL–HA internalized into macrophages and showed low cytotoxicity. In addition, CNT–PL–HA did not induce an inflammatory response in macrophages as evidenced by the cytokine profiling and the use of image-based high-content analysis approach in contrast to non-modified CNTs. In addition, systemic administration of CNT–PL–HA into healthy C57BL/6 mice did not alter the total number of leukocytes nor increased liver enzyme release as opposed to CNTs. Taken together, these results suggest an immune protective mechanism by the PL–HA coating that could provide future therapeutic benefit.

1. Introduction

Due to their outstanding properties, carbon nanotubes (CNTs) have emerged as promising nanomaterials in nanomedicine as both drug delivery vehicles and diagnostic tools [1–5]. The great interest in CNTs for biomedical applications derives from their unique structure and properties, which can be potentially exploited in a broad range of biomedical applications. CNTs have intense and unique Raman scattering, enabling easy detection in a variety of environments [6]. Their high aspect ratio enables to enhance the specificity and/or the potency of the particles by binding more biological species per particles [7]. Due to their hydrophobic nature, insoluble chemotherapeutic agents [8] and some proteins [9] adsorb spontaneously to CNT's sidewall and enable binding of functional groups. CNTs have been shown to cross the cell membrane in mammalian cells [10], and therefore have been used as transfection reagents in delivering nucleic acids such as small interfering RNAs (siRNAs) into a broad range of cells [11,12]. Many biological applications using CNTs have emerged in recent years. CNTs were used as protein carriers in transporting cytochrome c and its functionality was demonstrated by induction of cell apoptosis [9]. In another study, enhanced tumor suppression in mice was shown. The chemotherapy Paclitaxel was conjugated to polyethylene glycol functionalized CNTs and used to treat breast cancer in a murine model. The particles have enhanced efficacy in comparison with commercial Paclitaxel formulation (Taxol) [13].

Short SWCNTs (less than 100 nm) were used as non-viral vectors for the delivery of oligonucleotides (synthetic oligonucleotides containing the DNA binding sequence of a transcription factor) against nuclear factor-κB (NFκB) to human macrophages [14]. Together with the efforts of using CNTs as drug delivery vehicles, there were also attempts to use CNTs as imaging agents. The chelate DOTA was attached covalently to CNTs and was loaded with ^{111}In

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for radiolabeling. Rituximab monoclonal antibody (against CD20 expressed on B cells and up-regulated in many types of lymphoma and leukemia) was also attached to CNTs, and thus, a target specific contrast agent was constructed [15]. To exploit all unique properties of CNTs, their hollow tube was filled with metal halides that were used as radioprobes (tracked with single photon emission computed tomography) [16]. The primary and most critical step when trying to harness CNTs into biomedical applications is to overcome their insolubility in aqueous solutions such as water, buffers, growth media and sera. This poor-water solubility represents the main hurdle in utilizing CNTs in biomedical applications. Moreover, pristine CNTs were shown to have toxic effects when applied *in vitro* and *in vivo*. But it was also shown that properly functionalized CNTs have reduced toxic effects [17]. Two approaches for functionalization are employed for CNT modification: covalent and non-covalent. The benefit derived from non-covalent functionalization is that it does not interrupt the atomic lattice, and hence, the electronic properties of the tubes remain intact, whereas the benefit of the covalent method allows stable functionalization. In this respect, covalently functionalized SWCNTs exhibit reduced Raman absorption cross-sections [18]. In order to solubilize CNTs, various methods were developed to non-covalently functionalize CNTs including the use of surfactants, polymers, nucleic acids, peptides and proteins. In principle, all methods add soluble groups to the backbone of the CNTs and thus facilitate solubility. The attachment of relatively large functional groups is required to solubilize CNTs. Herein, we devised a strategy that utilized hyaluronan (HA) with its outstanding solubility characteristics as well as biocompatibility, as the functionalization agent on the surface of SWCNTs. HA, composed of repeating disaccharide units of D-glucuronic acid and D-N-acetylglucosamine linked *via* alternating β-1,4 and β-1,3 glycosidic bonds, is a high molecular weight glycosaminoglycan, and it is ubiquitously present in the extracellular matrix. It has many biological roles, including maintaining the extra cellular matrix (ECM) architecture and water retention, cell motility, migration and proliferation regulation, and cell adhesion and activation, and in tumor metastasis [19,20].

In this study, CNTs were conjugated to HA using phospholipids as the linking arm between HA and CNT. These particles have very good stability in aqueous solutions as reinforced by their zeta potential analysis. The effect of the structure of the phospholipid's hydrophobic tail on its ability to disperse CNTs was evaluated. Particle structure was analyzed by scanning electron microscopy (SEM) and by transmission electron microscopy (TEM). To confirm that these new nanotubes can be used in biological settings, their toxicity *in vitro* and *in vivo* was assessed using proliferation assays, high-content analysis and cytokine induction profile in cell lines along with measuring complement activation in human sera, leukocyte counts and liver enzyme release in healthy mice during a single and multiple intravenous administrations.

2. Materials and methods

2.1. Conjugation of phospholipids to hyaluronan

The carboxyl groups of HA and the primary amines of the phospholipids (phosphatidylethanolamine; PE) were exploited for conjugation. Using amine-coupling chemistry these two molecules were cross-linked. HA (750,000 kDa, Lifecore Biomedical, LLC (MN, USA)) was dissolved in doubled distilled water (DDW) to a final concentration of 5 mg/ml (HA solution). DPPE (Avanti Polar Lipids, Alabaster, AL, USA) was dissolved in ethanol (96%) at 1 mg/ml at 60 °C (DPPE solution). HA solution was activated for 15 min with carbodiimide (EDAC, 0.2 M) and sulfo-N-hydroxysuccinimide (NHS, 0.3 M) both purchased from Sigma-Aldrich Co. (St. Louis, MO, USA) at room temperature. Activated HA solution and DPPE solution were mixed at 1:1 ratio (v/v) and incubated for 2 h at 60 °C. The liquid was evaporated completely to discard the ethanol and the remnant was resuspended in DDW (equal volume). The aqueous solution was centrifuged at 300,000 *g* for 1 h and these washings were repeated three additional times to remove large HA aggregates.

2.2. Self-assembled CNT–PL–HA

1 mg of pristine CNTs (pCNT) (S-Purified Single-walled Nanotubes) as well as carboxylated carbon nanotubes (CNT–COOH), 50–70% carbon basis, D × L 1.2–1.5 nm × 2–5 μm, bundle dimensions were purchased from Sigma-Aldrich (St. Louis, USA).

pCNTs were suspended in 1 ml of the PL–HA solution as detailed above and sonicated in an ultrasonic water bath (Cole-Parmer, 100 W, 42 kHz) for 1 h. The solution was then washed three times to remove aggregates (10 min, 5000 g). To remove the excess HA–PL, the CNT suspension was centrifuged at 200,000 *g* for 45 min and the pellet was recovered. The final washing step was repeated and the pellet was suspended in DIW or PBS or any desired media (at this stage the concentration of the final solution could be determined) by spectral analysis in 808 nm. The suspension was sonicated again for 1 h and washed once (10 min, 5000 g) to remove non-dispersed material.

2.3. Phospholipids and quantitative analysis

Three different phospholipids (DOPE, DLPE and DPPE), all purchased from Avanti Polar Lipids Inc. (Alabaster, AL, USA) were covalently attached to HA to assess their ability to disperse SWCNT. All PL–HA conjugates were prepared as described and the absorbance at 808 nm (Cary-5000) of the suspensions (CNT–PL–HA) was measured to determine CNT concentration. Phospholipids were assayed by the lipid mass, modified Barttlet assay as previously described [21–23].

2.4. CNT structure analysis by electron microscopy

High-resolution Scanning Electron Microscopy (HRSEM-JEOL JSM-6700) and Transmission Electron Microscopy — TEM (JEOL 1200EX) were used for morphological assessment of the carbon nanotubes. Pristine CNTs were suspended in 1% Tween 80 solution, whereas the PL–HA counterparts were suspended in PBS. For HR-SEM, samples (0.1 mg/ml) were air dried on a silicon wafer and coated using a chrome sputter. For TEM, samples (0.1 mg/ml) were stained with uranyl acetate (2% in water) on a carbon-coated copper grid.

2.5. CNT structure analysis by Atomic Force Microscopy

AFM imaging and analysis was performed on a JPK NanoWizard III AFM system (JPK Instruments AG, Berlin, Germany), with tip scanning. Intermediate contact (tapping) mode was used. The AFM probe used was that of MicroMasch, NSC15/AlBS — a quite rigid, standard tapping mode probe, made of silicon, with Al coating on the cantilever backside. The resonance frequency was around 300 kHz and the line rate used was 0.5 Hz to 1 Hz, and 512 × 512 pixels.

Samples were prepared by placing a drop of stock solution of either pCNTs or CNT–PL–HA compounds on a freshly cleaved mica substrate.

2.6. Zeta potential measurements

The electrophoretic mobility of carbon nanotubes was measured using a Malvern ZetaSizer Nano ZS instrument at pH 6.7, 20 °C, with 10 mM NaCl. Each experimental result is the average of six independent measurements. CNT concentration of all the samples was 10 μg/ml.