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An investigation of the carbon nanotube – Lipid interface and its impact upon pulmonary surfactant lipid function

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ABSTRACT

Multiwalled carbon nanotubes (MWCNTs) are now synthesized on a large scale, increasing the risk of occupational inhalation. However, little is known of the MWCNT-pulmonary surfactant (PS) interface and its effect on PS functionality. The Langmuir–Blodgett trough was used to evaluate the impact of MWCNTs on fundamental properties of PS lipids which influence PS function, i.e. compression resistance and maximum obtainable pressure. Changes were found to be MWCNT length-dependent. 'Short' MWCNTs (1.1 μm, SD = 0.61) penetrated the lipid film, reducing the maximum interfacial film pressure by 10 mN/m (14%) in *dipalmitoylphosphatidylcholine* (DPPC) and PS, at an interfacial MWCNT-PS lipid mass ratio range of 50:1 to 1:1. 'Long' commercial MWCNTs (2.1 μm, SD = 1.2) caused compression resistance at the same mass loadings. 'Very long' MWCNTs (35 μm, SD = 19) sequestered DPPC and were squeezed out of the DPPC film. High resolution transmission electron microscopy revealed that all MWCNT morphologies formed DPPC coronas with ordered arrangements. These results provide insight into how nanoparticle aspect ratio affects the interaction mechanisms with PS, in its near-native state at the air–water interface.

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1. Introduction

Multi-walled carbon nanotubes (MWCNTs) are used extensively in commercial applications due to their extraordinary properties. Worldwide production of MWCNTs is now on the scale of several thousands of tonnes per year [1]. As the scale of synthesis increases, so does the inhalation risk to the people that work with them. Once inhaled, individual MWCNTs and small (<1 μm) CNT agglomerates can penetrate deep into the lung, and reach the subpleural region [2,3]. The epithelial surface of the respiratory tract is coated in pulmonary surfactant (PS), which plays a crucial role in reducing the surface tension of the alveolar air-liquid interface to prevent alveolar collapse during breathing and modulates innate immune defence. Inhaled MWCNTs that deposit in the alveolar region following inhalation will interact with PS and other lung secretions, before interacting with either alveolar macrophages or the alveolar epithelial cells [4,5]. It is therefore vital to improve our understanding of the interface between MWCNTs and PS components, as it will directly impact on PS function and the fate of MWCNTs after inhalation.

PS is synthesised by alveolar type II epithelial cells and consists of ~90% lipids and 10% proteins [6]. The majority of the PS lipids (80–90%) are phospholipids, the most abundant being phosphatidylcholine, of which ~50% is saturated dipalmitoylphosphatidylcholine (DPPC) [7]. DPPC is a polar molecule with a hydrophobic end of two saturated 16-carbon fatty acid chains and a hydrophilic end of a phosphate group with a quaternary amine group. It is responsible for reduction of the surface tension in the lungs to near-zero (equivalent to a surface pressure of 70 mN/m) [7].

Interactions between nanoparticles and DPPC or PS can be quantified using the Langmuir–Blodgett trough (LBT) which measures the behaviour of an insoluble film on the surface of a liquid during compression and expansion. Pure DPPC has been

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extensively studied using the LBT, often as a PS model, and undergoes phase transitions necessary for regulation of lung surface tension [8,9]. DPPC films display a transition between the solid liquid-condensed phase and the fluid liquid-expanded phase, which appears as a plateau in the isotherm. At the transition, the surface film consists of discrete condensed domains [10]. Once the monolayer is compacted into the condensed phase it becomes almost incompressible, indicated in the isotherm by a steep slope. When the monolayer is compressed past its limiting area, collapse occurs; at a certain surface density, the surface pressure can no longer increase and material is lost from the interface in an irreversible manner [11].

The monolayer behaviour of natural PS has also been characterised. PS is split into two fractions by differential centrifugation. The large aggregate fraction is used for monolayer studies. It contains phospholipids, tubular myelin, lamellar bodies, large vesicles and three of the surfactant proteins, SP-A, SP-B and SP-C [12]; of which the latter two are important for maintaining the surface activity of lung lining fluid. The small aggregate fraction consists mainly of small vesicles, less surfactant protein and is functionally inferior to the large aggregate fraction [13]. In isotherms of the large aggregate fraction, the primary characteristic feature is a squeeze out plateau, seen around 40–45 mN/m [14]. During compression to high surface pressures, the PS film becomes selectively enriched in DPPC, accompanied by "squeeze-out" of other constituents [15].

A number of studies have utilised LBTs to measure the impact of nanoparticles on the biophysical functionality of lung surfactants or associated models. 1wt% hydroxyapatite nanoparticles (~67.8 nm diameter) significantly reduced the compressibility of DPPC films, reducing the monolayer area by ~40% after 5 days exposure [16]. Subphase addition of 1wt.% silica nanoparticles (30 nm diameter) formed complexes with DPPC films and shifted the isotherm to higher molecular areas by 10% [17]. 130 nm poly(organosiloxane) nanoparticles rendered an 80:20:0.4 mol% DPPC/DPPG/SP-C model PS film incapable of increasing pressure past ~ 50 mN/m at a PS:nanoparticle mass ratio of 10:1 [18]. Eicosane, an alkane found in diesel exhaust particles, reduced the collapse pressure of a film of 1 mM DPPC (743 μg/ml) in a concentration-dependant manner between 0.04 and 2.5 mg/ml (Eicosane:DPPC mass ratio of 0.05–3.4) from 70 mN/m to ~66 mN/m, and caused a 30% increase in mouse surfactant surface compressibility [19]. Maximum pressures were reduced by 4 mN/m when single walled carbon nanotubes with an aspect ratio range 125–300, complexed with polyamidoamine (PAMAM) dendrimers, were adsorbed into DPPC monolayers from a 47 μg/ml subphase [20]. However, nothing is known about the effect of as-produced or multi-walled CNTs on the function of PS.

The aim of this study was to quantify the interaction of MWCNTs with DPPC monolayers and the large aggregate rat PS lipids at the air–water interface, as a function of MWCNT aspect ratio. The influence of MWCNTs on the maximum pressure and compression resistance of the DPPC and PS films, on the domain structure of DPPC and the formation of a lipid corona on the MWCNT surface were investigated. These interactions are critical parameters influencing MWCNT inhalation toxicity and could disrupt physiological surfactant function, whilst altering subsequent MWCNT bioreactivity [21].

2. Materials and methods

2.1. Carbon nanotube materials

MWCNTs were supplied by Nanostructures & Amorphous Materials, Inc carbon nanotubes. CNT length measured by SEM were 1.1 μm, SD = 0.61 ('short' MWCNTs) and length: 2.1 μm, SD = 1.2 ('long' MWCNTs). Diameters measured by TEM were 25 ± 0.7 nm and 26 ± 0.6 nm respectively.

In house MWCNTs were synthesized by injection assisted chemical vapour deposition [22]. A precursor solution of 1.5wt% ferrocene in toluene was injected into a 50 mm diameter quartz tube inside a furnace (Lenton PTF 15/-/610 furnace (max operation 1450 °C)) held at 780 °C through a 200 °C pre-heater with a carrier gas flow of 2000 SCCM argon. CNT length was measured at 35 μm, SD = 19, and diameter 44 nm ± 2 nm.

To evaluate particle size, the MWCNTs were observed under TEM (JEOL 2100F) at an accelerating voltage of 200 kV and SEM (LEO gemini 1525 FEGSEM) at an accelerating voltage of 5 kV. For TEM, samples were dispersed in ethanol and then drop cast onto lacey carbon coated copper grids. For SEM, samples were dispersed in ethanol and drop cast onto silicon wafers.

2.2. Rat lung lavage method

Sprague Dawley rats (12–14 weeks old, 300–400 g) were purchased from Charles River. Rats were sacrificed by an overdose of sodium pentobarbital (200 mg/kg) and lavaged with 2 × 10.5 mL saline via a tracheal cannula. The bronchoalveolar lavage (BAL) was centrifuged at 219 g to pellet the cells and the supernatant was centrifuged again at 28,110 g for 1 h at 4 °C to separate it into large aggregate (LA) and small aggregate (SA) fractions. The pellet (LA) was re-suspended in 40 uL saline. BAL lipid components were extracted from the LA by a Bligh and Dyer extraction and total organic phosphate was measured following perchloric acid digestion for 1 h at 200 °C, by the method of Bartlett against a potassium phosphate standard curve [23].

2.3. Langmuir–Blodgett trough

All reported measurements were performed using a Langmuir trough (KSV Nima, UK) with a total operational area of 85.4 cm^2. This trough is equipped with two Delrin barriers allowing symmetrical compression and expansion of the interface. Measurements took place at room temperature (22 ± 1 °C). DPPC is typically used at room temperature ~20 °C [24–27] due to the transition from its gel phase to its liquid crystal phase occurring above body temperature at 41 °C. It has been shown that temperature variations between 20 °C and 37 °C do not change the collapse pressure of DPPC films [7].

DPPC monolayers were formed by dissolving DPPC (Sigma) in chloroform (Sigma, anhydrous, ≥99%, contains 0.5–1.0% ethanol as stabilizer) at a concentration of 100 μg/ml. For the DPPC films which contained carbon nanotubes, a stock solution was made with the addition of MWCNTs in powder form to DPPC solution, and was subsequently diluted. All samples were sonicated for 10 min (bath sonication, 45 W) prior to being spread uniformly throughout the air–water interface using a 50 μl microsyringe (Hamilton). The initial volume was controlled to be 90 μl and all films were left for 30 min to allow chloroform evaporation. Pulmonary surfactant monolayers were formed by Bligh & Dyer extraction of saline suspended pellets of the large aggregate fraction from Sprague Dawley rats [28]. Lipids were extracted from 20 μl of the LA saline suspension and then diluted to an organic phosphate concentration of 4 μg/ml with chloroform for the control (Sigma, anhydrous, ≥99%, contains 0.5–1.0% ethanol as stabilizer), or a solution of MWCNTs in chloroform. 10 μl of sample was added to the Langmuir trough. Pulmonary surfactant films were left for 30 min for chloroform evaporation. The sub phase was Millipore deionised water (resistance 18.2 MΩ m at 25 °C). The DPPC films were compressed and expanded four times before obtaining the isotherm data, to allow for the equilibration processes in the film during the experiment [29]. A barrier speed of 5 cm^2/min (equivalent to 0.13 $Å^2$/mol s^{-1}) was used.

2.4. Atomic force microscopy

For atomic force microscopy, DPPC monolayers were compressed to 30 mN/m and held for 15 min. A previously submerged silicon wafer was then vertically drawn through the monolayer at a rate of 3 mm/min using a pulley system driven by a rotary motor (Cruzet geared DC motor, 1.5 rpm 12 V). Samples were dried under vacuum prior to imaging. Atomic force microscopy (AFM) images were taken on a Bruker Multimode 8 and NanoScope V controller using tapping mode with silicon tips (spring k = 42N/m, PPP-NCH from Asylum Research). Images were taken at a scan speed of 0.5 Hz with 512 lines and box sizes of 1, 5, 10 and 15 μm^2. Images were analysed with Gwyddion software.

2.5. TEM of DPPC coated MWCNTs

TEM samples were prepared by incubating MWCNTs in DPPC, then drop-casting onto holey carbon TEM grids (Agar). The TEM grid was then negatively stained with 2 wt% uranyl acetate solution [30]. Bright field imaging was carried out at 200 kV on a JEOL 2100F and at 80 kV on a FEG Titan.

2.6. Statistical analysis

All quantitive experiements were repeated at least three times. Data was expressed as means ± standard error. Statistical analyses were carried out using one-way ANOVA in Origin 9.1. Differences were considered statistically significant when the p value was less than 0.05. All isotherms are representative of a typical dataset for each sample.

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