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Impact of Lung Surfactant on Wettability and Cytotoxicity of Nanoparticles

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Abstract

The importance of nanoparticle characterization for nanotoxicology has been extensively emphasized and it has been universally agreed that the most important parameters for characterizing nanomaterials are specific surface area and surface properties (chemistry, hydro-philicity/phobicity, charge etc). This study is proposing the use of enthalpy of wetting which depends on both specific surface area and surface properties, is easily measurable and proves to be highly relevant for predicting nanoparticles' dispersion state and their interaction with the lungs. It also shows the conditioning effect of the lung surfactant main component, DPPC on the surface of particles when used in concentrations which mimic pulmonary exposure more closely.

1. Introduction

Currently, there is an increased interest in the potential toxicity effects associated with the inhalation of nanoparticles (NPs). Beside size, one distinctive property of NPs is their tendency to agglomerate and form weakly bound (by electrostatic and van der Waals forces) or sintered collections of primary particles. The degree of NP agglomeration could influence their toxicity [1-5] and therefore, the state of NP dispersion in the medium used to suspend them is an essential parameter required for the interpretation of *in vitro* nanotoxicity studies. Different strategies to disperse NPs and a range of biocompatible dispersion media have been investigated and proposed [4,6,7]. Researchers agreed that is important that the dispersion medium mimics the components of the lung lining fluid essential to NP dispersion and reported that their concentration affects not only the dispersion of NPs but also their

toxicity [5,6]. It is therefore critical for the concentration of the dispersion agents to also mimic the *in vivo pulmonary* conditions more closely.

Once inhaled, particles deposit in the hypophase where they adsorb the pulmonary surfactant on their surface and then interact with the epithelial cells. The lung surfactant is produced by the *alveolar type II cells* and coats the alveolar surfaces with a lining layer of lamellar liquid crystals (10-20 nm thick) formed by bilayers of long-chain phospholipids (90 wt%) stabilized by specific proteins (SP-C and SP-B,10 wt%) in the aqueous media [8,9,10,11]. The lamellar bodies maintain the function of the lungs: facilitation of gas exchange and prevention of alveoli collapse by reducing the lung air interfacial tension [12]. Dipalmitoylphosphatidylcholine (DPPC) together with proteins SP-B and SP-C are the main contributors to the surface activity of the pulmonary surfactant [13] and a large number of studies used DPPC as a surrogate of lung surfactant [1,4-6,14-24].

Through their adsorption on solid surfaces, surfactants can modify their wettability [25, 26]. Wettability of NPs is particularly important for understanding nanoparticle aggregation, dissolution and bioaccumulation [27]. Surface wettability, the degree to which a fluid spreads on a solid surface, depends on the nature of the surface and the fluid involved. Used in concentrations higher than the critical micelle concentration, surfactants adsorb as monolayers on hydrophobic surfaces/particles (i.e. carbon, polymers, etc.) and form bilayers on hydrophilic ones (i.e. metal, metal oxides, glass, ceramics) [28, 29, 30]. This is also the case of the zwitterionic surface agent DPPC which in high concentrations, adsorbs on hydrophilic surfaces as multiple bilayers (5-6 nm in thickness) which trap a 2 nm layer of water in between them [31]. The complete DPPC bilayers expose the hydrophilic moieties and provide polar, water 'wettable' surfaces.

The lamellar liquid crystals formed by the lung surfactant in hypophase contain abundant amounts of DPPC and enables adsorption of complete DPPC bilayers on hydrophilic particles and monolayers on hydrophobic particles. Regardless of the wettability of particles, through its adsorption on their surface, DPPC renders them hydrophilic (Figure 1) and consequently influences their dispersability and surface charge.

Numerous studies investigated the dispersion and toxicity of fine and ultrafine particles in DPPC or BAL (brochoalveolar lavage) aqueous media. The concentration of the DPPC/ BAL varied widely and generated potentially conflicting outcomes of both, dispersion state and toxicity of particles as it has been emphasized by several authors [1,4, 7, 32]. Wallace and al. [6, 14- 22] investigated and revealed the importance of employing high DPPC concentrations in particle toxicity studies. Other authors [5,23,33] who followed Wallace's recommendation reported similar toxicology results. Despite the evidence of these reported findings, the majority of published research used diluted DPPC/BAL dispersion media which did not promote a complete phospholipid mono (on hydrophobic NPs) or

bilayer (hydrophilic surface) coverage. An incomplete DPPC monolayer/bilayer coverage alters the wettability of the particles and it can convert hydrophilic particles into hydrophobic. This explains the outcomes of many studies which reported that DPPC/ BAL dispersion media led to particle agglomeration, sedimentation and various contradictory toxicity effects.

Wetting is primarily an enthalpy driven process and the *enthalpy of wetting* is directly related to parameters which have been linked to toxicity of NPs such as: surface area, surface charge, chemistry and solubility and has the potential to be a valuable characterization parameter [34,35].

This study has investigated how DPPC adsorbs on the titania P25 NPs and influences their dispersion and cytotoxicity. It also introduces a novel characterization parameter, *the enthalpy of wetting* of NPs and explores its potential to predict the NPs dispersion and interaction with biological systems.

2. Materials and methods

2.1. Test particles

Aeroxide P25 titania NPs were supplied by Evonik Industries AG. The primary particle size, specific surface area and tamped density supplied by the manufacturer in the product information sheet, are listed in Table 1. The phase composition also shown in Table 1 was calculated from XRD analysis. The pH of point of zero charge (PZC) of titania P25 NPs measured in deionized water (DI) was 6.7.

Table 1: P25 Titania NP parameters

	Mean primary particle size diameter (nm)	BET Specific surface area (m^2 g^{-1})	Tamped density (g l^{-1})	Composition (wt.%)
Aeroxide P25	21	50±15	approx. 130	80 % anatase & 20 % rutile

2.2. DPPC coating of P25 titania nanoparticles

1,2-dipalmitoyl- *sn*-glycero-3-phoshocholine (DPPC) ≥99% (M=734.04) was purchased from Sigma Aldrich, UK.

The coating of the NPs with DPPC followed the procedure proposed by Wallace and collaborators [6]. The surfactant dispersion was prepared at 37 °C by ultrasonically mixing DPPC in physiological saline (PSS) at 1.25 mg DPPC/1 ml PSS. Although surface active, lecithin has very low solubility in water. Titania P25 NPs were ultrasonically mixed into the surfactant dispersions in 1 mg NPs/1.25 mg DPPC/1 ml PSS and gently stirred in an incubator at 37 °C for one hour. NPs were centrifuged (4025 G-force) and the supernatant decanted. Additional DI water was added to the pellet and the centrifuge-washing

step repeated three times. The DPPC coated P25 NPs were freeze-dried to a powder. This procedure ensured that the titania NPs were fully covered in lipid but only a *single bilayer* remained as showed by the Raman analysis and wettability results.

To investigate the dispersability of the pristine and DPPC coated NPs in DI water (Millipore Nanopure Water), the dispersions were prepared in three concentrations: C1=1000 µg/ml, C2=100 µg/ml and C3=10 µg/ml. NP dispersions in C1 were sonicated for 15 minutes using a bath sonicator before dilution to C2 and C3. Vortexing at maximum power for 15 seconds was employed before dilutions and the measurement of particle size distribution and surface charge. The pH of the NP dispersions in DI water was approximately 7.

2.3. Characterization of NP dry powders

2.3.1. BET specific surface area

Specific surface area measurements have been carried out on a Micromeritics Gemini V BET surface area analyser. Prior to measurements, samples of 150-200 mg have been degassed in nitrogen atmosphere overnight at 25 °C. Two measurements were carried out for each sample.

2.3.2. Raman spectroscopy

Raman spectroscopy analysis was performed using a microRaman spectrograph (Horiba LabRAM Aramis integrated confocal system 460 mm focal length) with an internal He-Ne laser 633 nm and 20 mW output power. The laser source was focused on the samples through a long working distance 50x objective to a spot diameter of 2 µm. The acquisition time for Raman spectra was 10-20 minutes depending on the strength of the Raman signals, until a satisfactory signal-to-noise ratio was achieved.

2.3.3. Microcalorimetry

The heat of wetting/dewetting of NPs was measured with the TA Instruments Relative Humidity (RH) Perfusion Microcalorimeter equipped with the TAM III thermostat. The microcalorimeter is a heat-flow calorimeter of the twin-type which uses a 20 ml ampoule. It was preferred to the nanocalorimeter because the ampoule size permits a larger contact area of the water/solvent vapours with the nanopowder and can therefore reveal smaller differences in the wettability of samples.

The RH was kept constant at 10% until a stable heat flow was reached (signal within the range of -1 to +1 µW). The RH was then increased in a linear ramp from 10% to 90% over the following 10 hours and maintained at 90% for 10 hours. During the increase of humidity, the moisture interacts with the sample and produces a heat flow, called *heat of wetting*. The TAM III was housed in a thermostatic

room maintained at 25±1˚C and it was calibrated with empty stainless steel ampoules before the experiments. The measurements were carried out on 35-50 mg of samples and were repeated twice.

2.4. Characterization of NP dispersions

2.4.1. Particle size distribution and charge

The hydrodynamic size distributions of the NP dispersions were measured with the Malvern Zetasizer and CPS Disc Centrifuge (*CPS Disc Centrifuge* Model DC24000, CPS Instruments, Europe) which are Dynamic Light Scattering (DLS) techniques. In the CPS Centrifuge, dispersion samples of 300 µl were analyzed at 12000 RPM. The *ZetaSizer Nano ZS* (Malvern Instruments, UK) equipped with MPT-2 Multipurpose Titrator was employed for measurements of hydrodynamic particle size distribution, surface charge (zeta potential) and point of zero charge (PZC).
The titania dispersions were prepared in DI water rather than other biological media to be able to compare the particle size and surface charge results with the wettability values (heat of wetting/dewetting of water vapours). Prior to the size and zeta potential measurements, titania dispersions were sonicated for 15 minutes using a bath sonicator and vortexed for 15 seconds before each dilution. All measurements were carried out at 25 ˚C. Repeatability of all hydrodynamic size and zeta potential was checked more than three times.

2.5. Cell culture

A549 cells from a human lung adenocarcinoma with alveolar type II phenotype were obtained from Salisbury, UK (ECACC #8601284). Cells were cultured in RPMI 1640 with 25 mM HEPES and Glutamax (Gibco #72400-021), 10 % FBS (ATCC #30-2021), 1% Penicillin-streptomycin (100x Gibco #15140-122), 0.5% Fungizone (100x Gibco #15290-018) and Tryspin-EDTA (10x Gibco #15400-054) in a humidified atmosphere at 37 ˚C.

A cytotoxicity dose–response curve was created for the different nanoparticles, in the presence or absence of DPPC, using the thiazolyl blue tetrazolium bromide (MTT) or lactate dehydrogenase (LDH) assay. The cells were seeded at a density of 100 x 10^3 cells/cm^3 and medium was removed 24 hours later. The cultures were exposed to 3.3, 10, 33, 100, 333, 1000, 3333 µg nanoparticles/ml. DPPC alone was dosed at a 10 fold lower concentration [36]. In these experiments, controls and negative controls were included as described in [36,37].

2.5.1. MTT assay

After 24 h of exposure, the medium was removed and cells were rinsed with HBSS. The MTT solution (0.5 mg/ml MTT in HBSS) was added for 3 hours. Thereafter, the MTT solution was removed, and 100 µl of DMSO was added. The optical density was read at 550 nm with 655 nm as reference wavelength, using a microplate reader (Bio-Rad). Cells not incubated with nanoparticles and/or DPPC served as 100% viability control (*n=6 wells in 2 separate experiments)*.

2.5.2. LDH assay

Cells integrity was assessed by determining the percentage of LDH retained by the cellular layer. For LDH assay, after incubation with particles, the medium was removed; the adherent cells were washed twice with PBS (200 mL) and then lysed using Triton-X (0.2%). The LDH activity of the medium (LDH medium) and the cell lysate (LDH cells) were determined spectrophotometrically monitoring the reduction of pyruvate. Cell viability was calculated according to the formula:

% viability = [LDHcells / (LDHcells + LDHmedium)] x 100 (*n=6 wells in 2 separate experiments)*.

3. Results and discussion

3.1. Adsorption of lung surfactant on NPs

The surface of P25 titania NPs dispersed in water is generally covered by hydroxyl groups [38]. Having a polar surface, titania NPs are hydrophilic and will adsorb at least a bilayer of DPPC on their surface. In the current study, the calculated amount of DPPC necessary to cover with a complete bilayer 1 mg P25 titania NPs was 0.32 mg (considering the surface area per DPPC molecule of 40 $Å^2$ and the specific surface area of P25 NPs of 52.58 m^2/g). To ensure the DPPC concentration was sufficiently high to form a complete bilayer on NPs, we started with the amount ratio recommended by Wallace [6] of 2.50 mg DPPC/1 ml PSS/1 mg NPs.

This DPPC amount was difficult to disperse in PSS and it was diluted down to 1.25 mg DPPC/1 ml PSS. 1 mg NPs was mixed in the phospholipid dispersion which contained four times the DPPC amount necessary for complete bilayer coverage. The excess DPPC was removed by multiple rinsing with DI water.

The recommendations of Wallace and al. for the DPPC/NPs wt. ratio were based on data from unpublished investigations. In earlier published studies of microsized silica particles, Wallace employed the wet phosphate assay to quantify the particle uptake of DPPC from PSS dispersions at 37 °C over a range of DPPC dispersion concentrations. Two types of silicate particles, quartz and kaolin with specific surface area values of 4 m^2/g and 13 m^2/g respectively were found to adsorb 50-60 mg and 150 mg lecithin/gram as multilayers [15,19].

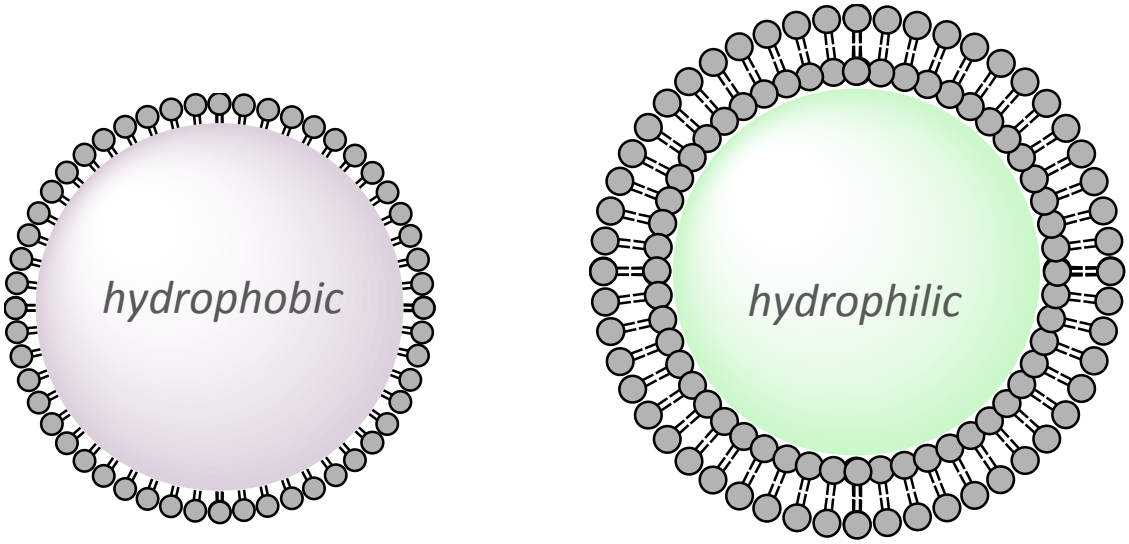


Fig. 1. Schematics of phospholipid adsorption as a monolayer on hydrophobic surface NPs (i.e. polymers, etc.) and as bilayers on hydrophilic surface NPs (i.e. metals, metalloids, oxides, etc.). The phospholipid coating renders the surface of all NPs hydrophilic (water wettable) when used in concentrations which mimic *in vivo* conditions.

The 2.50 mg DPPC/1 mg NPs wt. ratio recommended by Wallace [6] is indeed high but it is the amount required to assure a complete bilayer coverage of NPs such as polar (i.e. surface functionalized) CNTs because of their very high specific surface area (i.e. 2.2-3 mg DPPC are necessary to achieve complete bilayer coverage of 1 mg CNT with S_{SA}=490 m^2/g). The excess DPPC can be rinsed off from the coated NPs because the complete monolayer/bilayer is stable to water rinsing [6, 39].

The presence of the DPPC bilayer coating on NPs was tested with Raman spectroscopy. The Raman spectra for the DPPC coated P25 NPs, P25 NPs and DPPC powder are shown in Figure 2.

For DPPC powder, a representative spectrum similar to other published spectra [40] is shown in Figure 3 with band assignments. Spectral peaks can be found in the fingerprint region (600-1800 cm^{-1}) associated with choline symmetric stretch (718 cm^{-1}) and the lipid hydrocarbon acyl chains: (1060, 1100, and 1128 cm^{-1}) but the most intense bands in the spectrum are the C-H stretching region (2800-3100 cm^{-1}). The methylene vibrations at 2845, 2880, 2930 are sensitive to conformational changes as well as intermolecular interactions of the alkyl chains of lipids and therefore has been much research focused on their interpretation. The Raman spectra for DPPC-P25 NP as compared to DPPC powder show a change in intensity ratio for the 2850/2880 cm^{-1} bands. The intensity ratio, I_{2880}/I_{2845} derived from these spectra, serves as an index of the strength of the lateral interchain packing interactions in the bilayer [41]. The

intensity ratio value for DPPC-P25 is 1.04 similar to that found by other researchers for supported DPPC bilayers on SiO_2 NPs with a diameter of 30-40 nm [42].

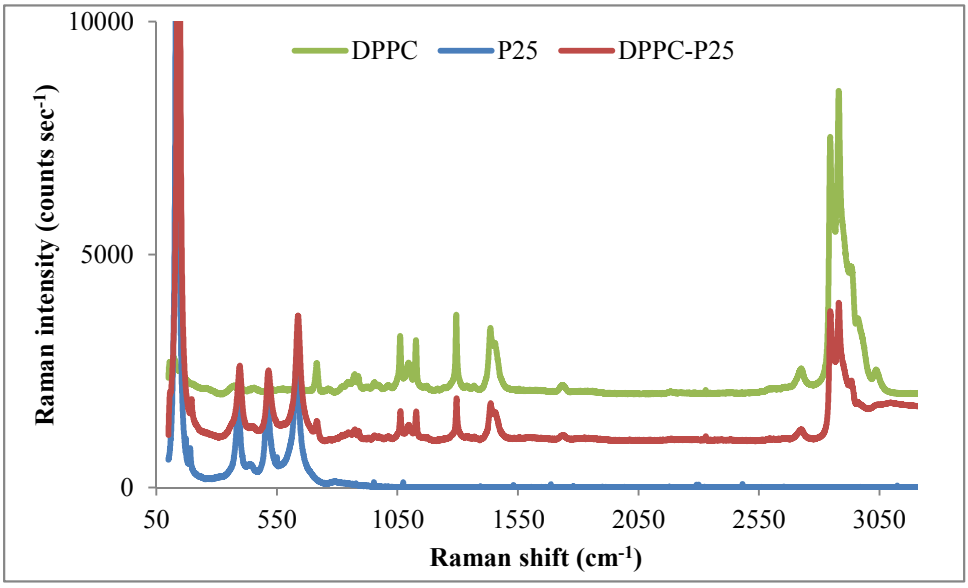


Figure 2. Raman Spectra for P25 NPs, DPPC coated P25 NPs and DPPC powder

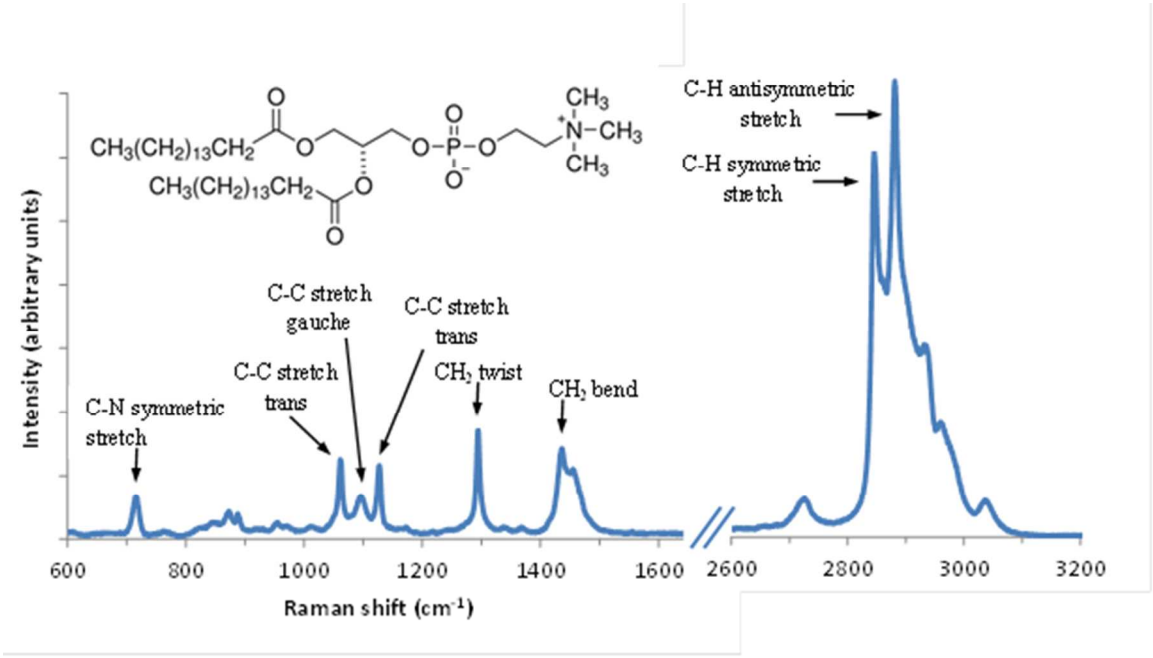


Figure 3. Raman Spectrum of DPPC powder, showing structure of DPPC and band assignments

3.2. Effect of DPPC coating on wettability, dispersability and surface charge of NPs

For solid surfaces, the most popular parameter used to describe wettability is the contact angle. For powders, two main methods have been traditionally used for determining the contact angle: measurements on a compacted powder surface and of the penetration into loosely packed beds but because of the irregular geometry of the systems both present problems [43,44]. The penetration experiment uses the Washburn equation which has been shown to be flawed [45] and in the case of compressed powder, the effect of compression affects the values of the contact angle. A newer, simple approach was proposed by Shanker [46], in which the powder is glued to an inert support but this method also has its practical drawbacks. Recently, two novel techniques for determination of contact angles of NPs were proposed: multi-angle single-wavelength ellipsometry [47] and freeze-fracture shadow-casting cryo-scanning electromicroscopy [48]. These methods also have shortcomings and are yet at the experimental stage.

Isothermal microcalorimetry, which measures the heat changes associated with the wetting process, is a sensitive and straightforward way to measure wettability of powders. Measurements are carried under controlled environmental conditions and can also be carried out for hydrophobic powders. Generally, the heats of immersion in water are high for the hydrophilic surfaces (indicating a strong interaction with water molecules) and low for the hydrophobic ones [49].

Enthalpy of wetting is the heat of wetting (measured with isothermal calorimetry) divided by the specific surface area of the powder and depends on the nature of the surface (chemistry and roughness) and the dissolution (solubility) in the wetting fluid. For a number of inorganic oxides, a linear relation has been found to exist between the heat of wetting and the point of zero charge (PZC) [34,35].

Figure 4 shows the thermograms for wetting and de-wetting by water of the surface of P25 and DPPC coated P25 NPs. The humidity has been increased and decreased from 10 to 90 %.

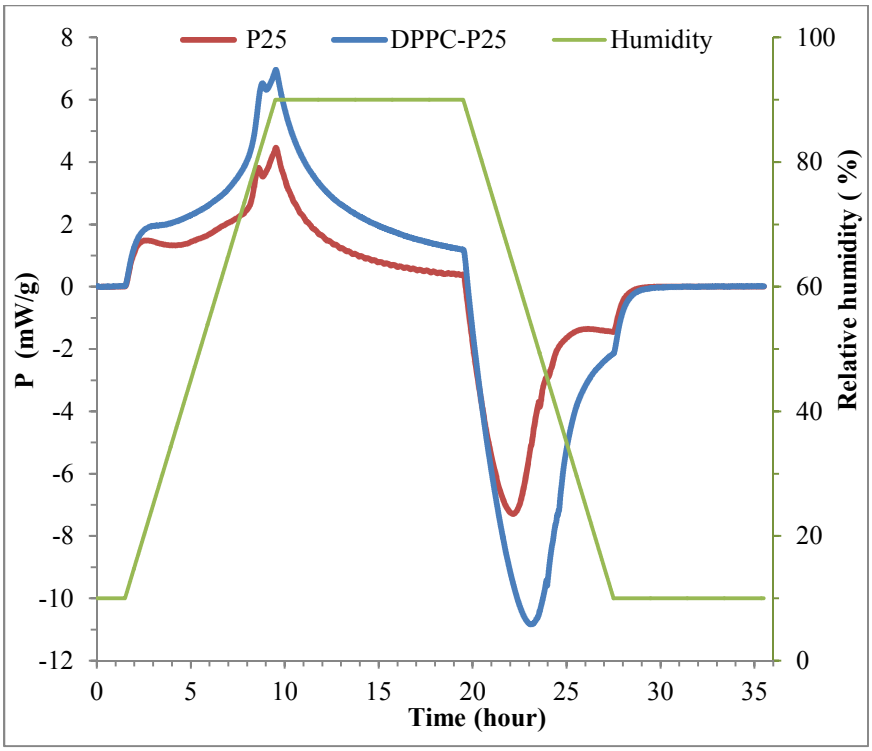


Figure 4. Thermograms for wetting/dewetting of P25 and DPPC coated P25 NPs

Table 2 shows the measured values of the heat of wetting/de-wetting with water and the BET specific surface area of the P25 and DPPC coated P25. All measurements were carried out at 25 °C and were repeated at least once.

Table 2: Surface area, heat and enthalpy of wetting of P25 and DPPC coated P25 NPs

	P25	DPPC-P25
BET Specific Surface Area (m^2/g)	52.58 ±3	14.54 ±1
Heat of Wetting/Dewetting $-\Delta h_w/\Delta h_{dw}$ (mJ/g)	91.8/91.99 ±2	179.5/173.6 ±1
Enthalpy of Wetting $-\Delta H_w$ (mJ/m^2)	1.7	12.3

Both samples show an exothermic heat flow (negative heat of wetting) during moisture adsorption (increasing humidity) and endothermic heat flow during moisture desorption which indicates that the surfaces of NPs are hydrophilic. The heat of immersion enthalpy (absolute value) and the surface free energy increase with the hydrophilicity of the sample. By integrating from a 10% RH equilibrium through to 90% RH and reversing to 10% RH equilibrium there is a significant difference in the moisture interaction (wettability) between samples. The DPPC-NPs have larger negative values of heat than the non-coated NPs which indicates that the DPPC coating increased the hydrophilicity of P25 NPs. Published work which measured the surface free energy and its components to study wettability of spin coated DPPC applied on hydrophilic materials (slides of glass, silica and mica) reported similar findings. The concentration of the DPPC was found to exert a major influence on the wettability of the coating. The hydrophilic character of the coating decreased when DPPC concentrations were up to 0.5 mg/ml and it increased and levelled off when 2-2.5 mg/ml was used. The advancing contact angle of water in the second case was $10\pm2.6^{\circ}$ which means that water almost completely spreads on the DPPC coating [50].

The enthalpy of wetting is calculated by dividing the measured heat of wetting by the BET specific surface area.

The measured BET specific surface areas for the pristine and DPPC coated P25 NPs are shown in Table 2. It is known that the surface area and porosity of NPs can change as a result of adsorption of biomolecules or agglomeration [27]. Our results show that the surface area of DPPC-P25 NPs has been substantially decreased by the DPPC bilayer adsorption on P25 NPs. BET surface area measurements were also carried out on pristine and DPPC coated *rutile* titania NPs (our unpublished data) and a similar degree of reduction in specific surface area was recorded. The particle size distribution data which will be presented later showed that the agglomeration of the NPs did not contribute to the size decrease because the particle size distributions of DPPC-P25 dispersion in DI water indicate much lower average sizes and polydispersity than the pristine P25 NPs. Therefore, we considered that this decrease in specific surface area can be due to two main factors. First, in the calculation of the specific areas of both, the pristine and DPPC coated P25 NPs, the same tapped density value of 1.3 g/cm^3 (supplied by the manufacturer) was used because the tapped density value for DPPC-P25 NPs wasn't available. However, the DPPC coating increases the size of NPs by 30% (21 nm + 10 nm DPPC bilayer coating) and consequently, the density of the DPPC-P25 NPs must be smaller. For example, if the density of a P25 NP is ρ_{P25}= 3.8 g/cm^3 and the density of the DPPC coating ρ_{DPPC}=1.03 g/cm^3, the density of a DPPC-P25 NP will be reduced to half the density value of P25 NP ($\rho_{DPPC\text{-}P25} \cong 1.9$). This reduction of the DPPC-P25 NP density implies that the measured BET specific surface area is actually twice the value shown in Table 3.

The second factor which could contribute to the large decrease in specific surface area of DPPC-P25 NPs is the potential of the coating to cover/block the pores present on the surface of the P25 NPs. BET surface area depends on the amount of N_2 gas adsorbed in the pores of the NPs surface. P25 NPs have pore volume of 0.25 cm^3/g and pore size of 17.5 nm [51] and it is mesoporous according to IUPAC classification (size of pores 2-50 nm). By covering the pores and the adsorption sites on the P25 NPs, the DPPC coating can reduce the roughness and therefore, the specific surface area of NPs.

The hydrophilicity (water wettability) and the surface charge of NPs are important factors which influence their dispersability and colloidal stability in water. To further investigate the effect of the DPPC coating on the P25 NPs, dispersions of NPs in DI water were prepared in a range of three concentrations relevant to the toxicology studies. The surface charge of NPs and the size distribution of the dispersions were measured with the Zetasizer and the CPS centrifuge.

Tabel 3. Surface charge of NP dispersions in DI water in three concentrations

	Concentration (µg/ml)	Av. Zeta Potential at 22°C (mV)
P25	$C_1 = 1000$	-0.5
	$C_2 = 100$	-21.1
	$C_3 = 10$	-24.2
DPPC-P25	$C_1 = 1000$	-29.0
	$C_2 = 100$	-35.1
	$C_3 = 10$	-39.4

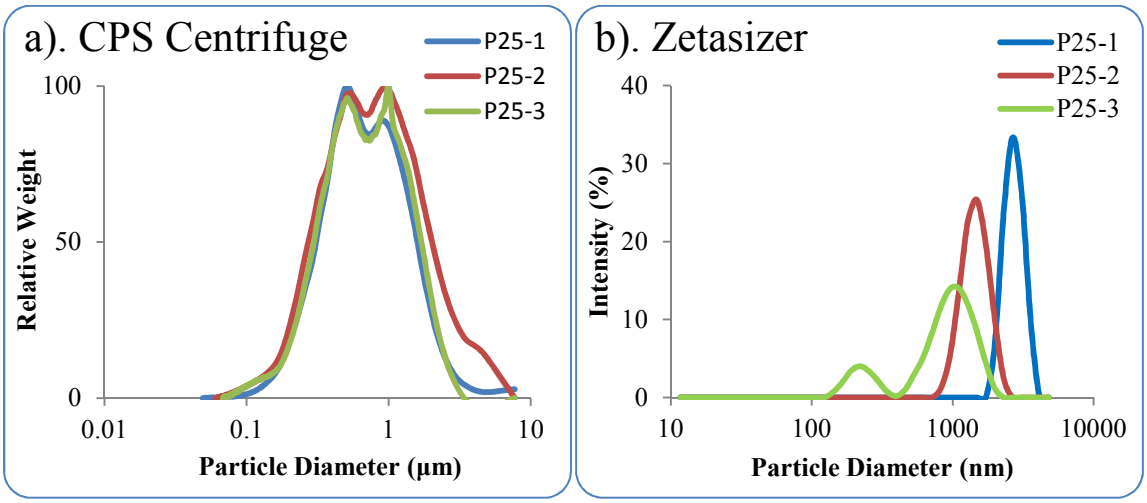
The zeta potential results listed in Table 3 indicate that the DPPC-P25 NPs have the largest measured negative surface charge values in all three concentrations. The pH of the dispersions was approximately 7 and all zeta potential values are at or above the threshold value for stable dispersions (-30 mV).

Figures 5a and 5c show the particle size distribution measured with the CPS centrifuge for the pristine and DPPC coated P25 dispersions in three concentrations. The CPS centrifuge results shown in Figure 5a indicate that P25 dispersions in DI water were broadly polydispersed (PSD spreading between 0.08-5 µm). The results measured with Malvern Zetasizer (Figure 5b) were not qualitatively acceptable because of the polydispersity of the samples.

The DPPC-P25 NP dispersions measured with CPS centrifuge (Figure 5c) and Malvern Zetasizer (Figure 5d) show low average sizes and narrow distributions for all three concentrations tested. In this case the Malvern Zetasizer results were qualitatively acceptable and the size distribution was more similar to

that measured by the CPS centrifuge. Both instruments measure the dispersions hydrodynamic diameter which is controlled by NPs agglomeration (function of zeta potential and ionic strength of dispersion media) in the aqueous system but also a strong function of primary particle size. Published research studies [38] which also employed the Malvern Zetasizer to investigate titania NP dispersions in DI water showed that even when the dispersions were stable (the zeta potential was 30 mV), the measured average hydrodynamic diameter was much larger than the primary particle size (the measured average hydrodynamic diameter of NPs with primary size of 6 nm was 67 nm).

Figures 5e and 5f compare PSDs measured with the CPS Centrifuge and Malvern Zetasizer of 0.1 mg/ml (C2) P25 and DPPC-P25 dispersions in DI water. The DPPC-P25 dispersion has a small average particle size and a narrow distribution while the P25 dispersion has large average particle size (forms large aggregates) and is broadly polydispersed. The Zetasizer distribution results for the P25 dispersions did not meet the quality criteria because the polydispersity index was too high (the size distribution for C2 dispersion was plotted in figure 5f for comparison only).



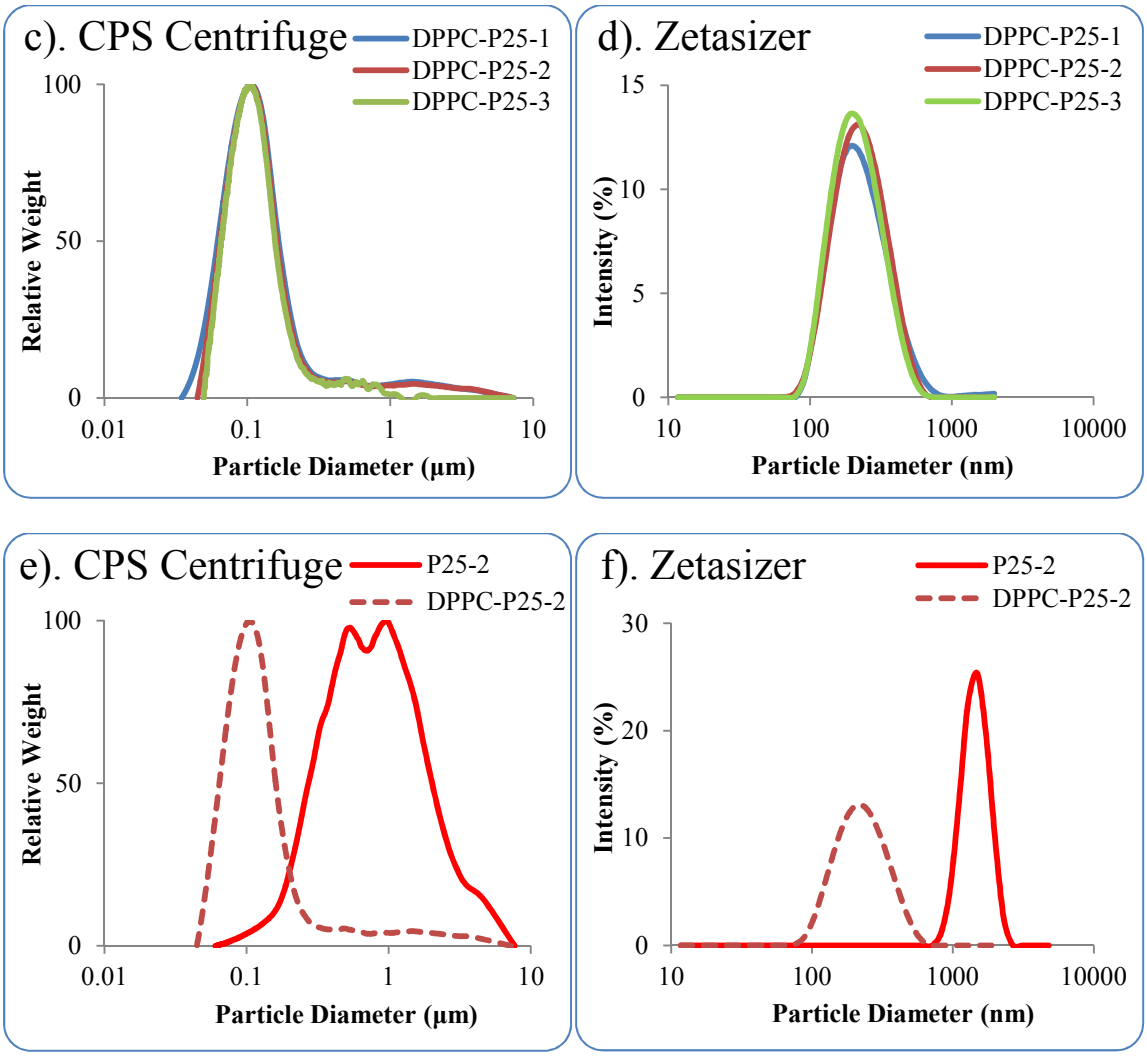


Figure 5. Particle size distribution of NP dispersions in DI water a). P25 C1=1, C2=0.1, C3=0.01 mg/ml dispersions measured with CPS Centrifuge, b). P25 C1=1, C2=0.1, C3=0.01 mg/ml dispersions measured with Malvern Zetasizer, c) DPPC-P25 C1=1, C2=0.1, C3=0.01 mg/ml dispersions measured with CPS Centrifuge, d). DPPC-P25 C1=1, C2=0.1, C3=0.01 mg/ml dispersions measured with Malvern Zetasizer, e). P25 and DPPC-P25 C2=0.1 mg/ml dispersions measured with CPS centrifuge and f). P25 and DPPC-P25 C2=0.1 mg/ml dispersions measured with Malvern Zetasizer

The particle size distribution and zeta potential results indicate that the DPPC coating increases the surface charge and reduces both, the average hydrodynamic diameter and polydispersity of the P25 NP dispersions, therefore generating a more stable NP dispersion. These results correlate very well with the heat of wetting values which also show a large increase in the wettability of P25 NPs when coated with a complete bilayer of DPPC.

3.3. Effect of DPPC coating on cytotoxicity of NPs

Figure 6 a and b show the 24 hours A549 cell viability versus nanoparticle concentration and the SDs of the pristine and DPPC coated P25 using the LDH and MTT assays.

The results show the effect of the DPPC coating on the NP cytotoxicity. One way Anova reveals that in the LDH and MTT assays, P25 NPs are significant different from DPPC-P25.

The toxicity of DPPC-P25 NPs is reduced in the entire concentration range tested and reached 25% (75% viability) at high NP concentrations (above 100 µg cm^{-1}). For the DPPC-P25 NPs there is a slight downshift in the viability curve for the top two concentrations but these doses are very high as the highest standard dose is typically 333 µg cm^{-2}.

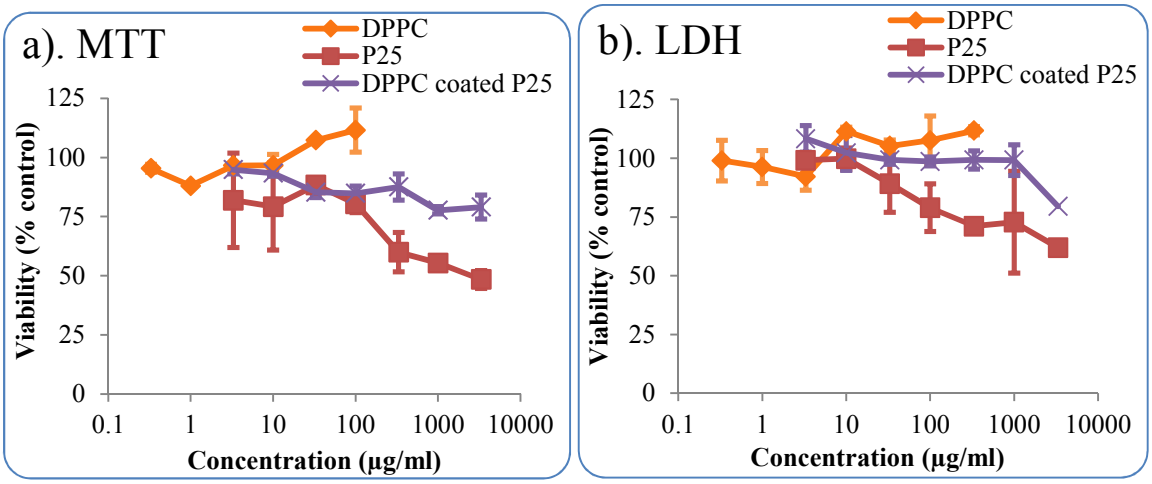


Figure 6. A549 cells viability exposed to pristine and DPPC coated P25 NPs a). MTT assay and b). LDH assay

Several studies employed DPPC concentration which enabled the complete coverage of NPs and demonstrated the protective effects of lung surfactant coating on toxic particles (silica etc.). Wallace and al. [15] showed that the DPPC treated quartz and kaolin particles incubated for 1 hour with erythrocytes suppressed the hemolytic strength of particles to background levels. Studies on pulmonary alveolar macrophages showed the DPPC fully suppressed silica NPs prompt cytotoxic, apoptotic, necrotic and genotoxic activity for 3 days [15,16,33,52] The interaction between DPPC and different types of silica dust has been suggested to modulate the toxicity of the particles through the molecular orientation of the adsorbed DPPC molecules. This may account for differences in pulmonary disease abnormalities [21]. The

artificial surfactant Survanta (FDA approved pulmonary surfactant extracted from minced cow lung with additional DPPC, palmitic acid and tripalmitin) protected alveolar macrophages *in vitro* and reduced lung injury *in vivo* without affecting neutrophil influx [53]. In the special case of crysotile asbestos, the prophylactic effect of the DPPC coating was found ineffective at inhibiting its toxicity [33] despite the reported ability to adsorb on its surface [54]. This finding reveals that the toxicity of crysotile asbestos may thus depend on parameters other than the surface properties such as the shape of particles.

The protective effect against toxic particle induced lung-damage is only temporary as research showed that subsequent removal of the prophylactic DPPC coating through secondary lysosomal or extracellular PLA-2 phospholipase enzymatic digestion restored the toxicity in 3-7 days [15,17-20,33,55]. These differences were ascribed to variations in the rate of digestion by lysosomal enzymes.

It was emphasized in toxicology studies that critical toxic events in mineral dust-induced fibrosis occur in cells in the alveolar interstitium rather than in the macrophages of the alveolar surface hypophase [56] and investigations should also be carried out on pulmonary alveolar interstitial cells [19]. Sager [1] reported that data from their laboratory indicate that improved dispersion of SWCNTs resulted in significant enhanced potency of aspirated material to induce interstitial fibrosis in mice.

Apart from the damage of lysosomes by the phagocytized cytotoxic particles another important reaction of the macrophages with particles is the production and release of reactive oxygen species (ROS). Hertog [5,23] and Foucaud [24] employed DPPC concentrations which according to our calculations enabled complete monolayer coverage of their studied hydrophobic NPs, SWCNT and CB respectively and reported an improved particle dispersion which was considered the reason for the increased ROS production in A549 cells and DCFT oxidation in MonoMac-6 human cells.
Numerous reported studies showed that dispersion of NPs (especially CNTs) is critical to their effective distribution in the lung. In line with these studies, our results revealed that P25 NPs coated with a complete DPPC bilayer become more hydrophilic and generate stable dispersions with an average hydrodynamic diameter of 110-190 nm. Published TEM images of aluminum particles inside an endosome of A549 cell from *in vivo* studies also showed NP aggregates with similar sizes (100-200 nm) [27]. This emphasizes the importance of conditioning the NP surface in toxicology studies to mimic pulmonary exposure more closely. Employing DPPC in concentrations which enable a complete coverage of NPs is an important step in achieving this aim. The establishment of standard dispersion media and protocols will enable researchers to achieve a degree of concordance and hence, could greatly benefit nanotoxicology research.

4. Conclusions

The enthalpy of wetting includes two NP parameters highly relevant to toxicology, wettability and surface area and along with surface charge can be used to predict their dispersability and stability in aqueous fluids and consequently, their interaction with biological systems.

On titania P25 NPs, the adsorption of a complete DPPC bilayer has had the following critical effects:

1) **Increased hydrophilicity of NPs and aided their dispersion in aqueous media.**
2) **Reduces the specific surface area of dry NPs;**
3) **Reduced the toxicity of NPs on A549 cells in 24 hour tests.**

To mimic the *in vivo pulmonary* conditions more closely and enable the replication of the surfactant *in vivo* action, nanotoxicology studies should employ the most abundant surface active component of the lung surfactant, DPPC in a DPPC/NPs wt. ratio which enables complete coverage of NPs. Following this testing strategy will hopefully lead to reaching consensus in cytotoxicity results.

The investigation of DPPC adsorption on P25 titania NPs has provided important information on their wettability, charge and dispersability in biological fluids with consequences on the lung cells uptake and toxicity. This study represents a first step in establishing a protocol for nanotoxicity testing and we recommend similar tests to be carried out on hydrophobic NPs along with investigating the effect of the relevant SPs on the DPPC adsorption and particle toxicity.

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