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Effect of pulmonary surfactant on the dissolution, stability and uptake of zinc oxide nanowires by human respiratory epithelial cells

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Abstract

Inhaled nanoparticles have high deposition rates in the alveolar region of the lung but the effects of pulmonary surfactant (PS) on nanoparticle bioreactivity are unclear. Here, the impact of PS on the stability and dissolution of ZnO nanowires (ZnONWs) was investigated, and linked with their bioreactivity *in vitro* with human alveolar epithelial type 1-like cells (TT1). Pre-incubation of ZnONWs with Curosurf[®] (a natural porcine PS) decreased their dissolution at acidic pH, through the formation of a phospholipid corona. Confocal live cell microscopy confirmed that Curosurf[®] lowered intracellular dissolution, thus delaying the onset of cell death compared to bare ZnONWs. Despite reducing dissolution, Curosurf[®] significantly increased the uptake of ZnONWs within TT1 cells, ultimately increasing their toxicity after 24h. Although serum, improved ZnONW dispersion in suspension similar to Curosurf[®], it had no effect on ZnONW internalization and toxicity, indicating a unique role of PS in promoting particle uptake. In the absence of PS, ZnONW length had no effect on dissolution kinetics or degree of cellular toxicity, indicating a less important role of length in determining ZnONW bioreactivity. This work provides unique findings

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on the effects of PS on the stability and toxicity of ZnONWs, which could be important in the study of pulmonary toxicity and epithelial-endothelial translocation of nanoparticles in general.

Keywords

epithelial uptake; nanoparticles; nanotoxicity; alveoli; Curosurf®

Introduction

ZnO nanowires (NWs) are at the forefront of application-driven nanotechnology (Djurisic et al., 2012). The high-sensing capability and high electron mobility of ZnONWs are explored for various biomedical applications, such as biosensors for intracellular measurements (Hahm, 2014), and for cancer detection and therapy (Hong et al., 2011, Mitra et al., 2012). Their antibacterial properties are also explored for the fabrication of medical devices to prevent infections, including antimicrobial facemasks and tissues (Okuyay et al., 2015). Meanwhile, inhalation of fibrous nanomaterials (NMs) has raised particular concerns for adverse health effects, due to their similarity to some types of asbestos fibers and the possibility of inducing mesothelioma (Donaldson et al., 2011). Therefore, inhalation of ZnONWs represents a key exposure route to investigate, both from the perspective of intentional (diagnosis/therapy) and unintentional (occupational exposures during production) scenarios.

Upon inhalation, the nanometer scale of ZnONW width would define their aerodynamic diameter (D_{ae}) and ultimately allow for preferential deposition in the alveolar region of the lung (Sturm and Hofmann, 2009). In the alveoli, where removal is dominated by relatively slow, macrophage-mediated clearance, build-up of ZnONWs would potentially result in high alveolar concentrations. Ultrafine ZnO is known to cause pulmonary inflammation in humans (Fine et al., 1997), while *in vivo* inhalation of ZnO nanoparticles (NPs) in rodents induced systemic inflammation (Chuang et al., 2014). The toxicity of ZnONPs has been reported for various *in vitro* mammalian systems (Vandebriel and De Jong, 2012) including human bronchial epithelial cells (BEAS-2B) (Xia et al., 2008). However, there is a paucity of research regarding the bioreactivity of ZnONWs in the lungs (Ahamed et al., 2011). In our previous work, ZnONWs were toxic to human monocyte derived macrophages (MDMs) at similar concentrations as $ZnCl_2$, and cell death correlated to a rise in intracellular Zn^{2+} concentrations (Muller et al., 2010). Consequently, there is an urgent need to further investigate the bioreactivity of ZnONWs with the pulmonary epithelium, in order to uncover their toxic potential, and enable the design of efficient systems for biomedical applications.

More importantly, the effects of the pulmonary surfactant (PS), which represents a first line of defense of the lungs against inhaled NMs (Creuwels et al., 1997), on the bioreactivity of NMs, have not been sufficiently investigated (Theodorou et al., 2015a). PS consists mainly of phosphatidylcholine (PC), about 50% of which is saturated, especially in the dipalmitoylated form (DPPC) (Creuwels et al., 1997). Following deposition of inhaled ZnONWs in the alveoli, the PS may promote their interaction with the underlying epithelium, through wetting forces that draw the particles into the PS and towards the

alveolar wall (Mijailovich et al., 2010). Any effects of the PS on the physicochemistry of inhaled ZnONWs must be examined, as these could impact their interaction with cells and tissues in the lung (Theodorou et al., 2014). We have previously demonstrated that silver nanowires (AgNWs) incubated with PS were coated by a phospholipid corona, which delayed their oxidative dissolution and inhibited aggregation (Theodorou et al., 2015a). However, to our knowledge, no data exist on the stability of ZnONWs in the PS, or their subsequent bioreactivity with alveolar epithelial cells.

Given the central role of dissolution in the bioreactivity of ZnONMs and the fact that several factors may affect dissolution (*e.g.* size, shape, pH, organic components) (Bian et al., 2011, Xia et al., 2008), characterization of ZnONWs at the local *point of exposure* in cellular microenvironment is essential in order to draw accurate conclusions about their bioreactivity *in situ*. The purpose of the present work is to examine the impact of PS on the stability of ZnONWs and the subsequent effects on their interaction with alveolar epithelial cells. Inductively Coupled Plasma–Optical Emission Spectroscopy (ICP-OES), dynamic light scattering (DLS), zeta-potential and analytical transmission electron microscopy (TEM) techniques were combined to investigate the dissolution kinetics and colloidal stability of ZnONWs. The effects of PS were compared to those of serum, to investigate whether they are unique to the PS or general to a particle corona. The cellular toxicity of ZnONWs with two lengths (“short”: S-ZnONWs, 30 nm × 1 µm; “long”: L-ZnONWs, 30 nm × 3 µm) was studied in relation to cell viability of human alveolar epithelial type 1-like cells (TT1) (Kemp et al., 2008). Alveolar epithelial type 1 cells (AT1) cover 95% of the total alveolar surface, and are therefore susceptible to inhaled nanosized particles. Alveolar epithelial type 2 cells (AT2) synthesize and secrete PS. As AT1 cells are not commercially available, a unique TT1 cell line was used, generated from immortalized primary human AT2 cells and showing characteristics of AT1 cells (Kemp et al., 2008). Recent findings also suggest that TT1, but not AT2 cells, govern uptake and potential translocation of inhaled NPs across the pulmonary epithelial barrier (Thorley et al., 2014). Our results were compared to exposure to ZnCl₂, as well as ZnONWs pre-incubated with a model PS. A combination of TEM and ICP-OES were used to monitor and quantify the amount of particle uptake by cells. Finally, the kinetic intracellular dissolution of ZnONWs and its correlation to dynamic cell death were investigated by confocal microscopy of live TT1 cells exposed to ZnONWs.

Materials & Methods

In this section, the main aspects of the methods are summarized. Detailed descriptions of the methodology are provided in the Supplementary Material.

Short and long ZnO nanowires (S-ZnONWs and L-ZnONWs), 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC) and RPMI-1640 tissue culture medium were purchased from Sigma-Aldrich, UK. Propidium Iodide (PI), Hoechst 33342 and FluoZinTM-3, AM were purchased from Invitrogen, UK. Curosurf[®] was kindly donated by Chiesi Pharmaceuticals, UK.

Endotoxin assays of as-received ZnONWs were performed using an LAL Chromogenic Endotoxin Quantitation Kit (Thermo Scientific, UK).

ZnONW dissolution and stability

ZnONWs (25 µg/mL) were incubated in pH 7 and pH 5 normal saline (NS) solutions, in a temperature-controlled dri-block incubator at 37 °C, for 0–48 hours, in the absence or presence of DPPC (a phospholipid), Curosurf® (a natural surfactant from porcine lungs) or 10% newborn calf serum (NCS; Invitrogen, UK).

The amounts of free Zn²⁺ ions released from ZnONWs were measured by Inductively Coupled Plasma–Optical Emission Spectroscopy (ICP-OES, Thermo Scientific, UK).

Bright field TEM (BFTEM), high resolution TEM (HRTEM) and high angle annular dark field scanning transmission electron microscopy (HAADF-STEM), combined with selected area electron diffraction (SAED) and energy-dispersive X-ray spectroscopy (EDX) were performed using a JEOL 2000, a JEOL JEM-2100F or an FEI Titan 80/300 TEM/STEM fitted with a Cs (image) corrector.

Dynamic light scattering (DLS) and ζ-potential measurements were performed using a Zetasizer Nano ZS (Malvern, UK).

TT1 treatment with ZnONWs

The immortalized AT1-like human alveolar epithelial cell line (TT1) was created from primary human alveolar epithelial type 2 cells (AT2) (Kemp et al., 2008) and cultured as described in the Supplementary Material, before being exposed to ZnCl₂ or ZnONWs.

To assess the effect of serum, S-ZnONWs were prepared in RPMI-1640 containing 10% NCS. To investigate the effect of PS, S-ZnONWs were incubated for 24 hours with a 1:1000 dilution of Curosurf® in RPMI-1640. The incubated ZnONWs were collected by centrifugation and re-suspended in serum-free RPMI-1640.

Cell viability (WST) and lactate dehydrogenase (LDH) assays

Cell viability was analyzed using the cell proliferation reagent 4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulfonate, (WST-1; Roche, UK), while lactate dehydrogenase (LDH) release was analyzed using the Cytotoxicity Detection kit PLUS (LDH; Roche, UK), both as described in the Supplementary Material.

Quantification of ZnONW cellular uptake cellular

TT1 cells were treated for 24 h with ZnCl₂ or S-ZnONWs (bare or pre-treated with Curosurf®) and the intracellular amounts of Zn²⁺ were determined by ICP-OES, following acid digestion.

Following treatment with S-ZnONWs (bare or pretreated with Curosurf®), cells were infiltrated with a Quetol-based resin and thin sections (90 nm) cut from the resin blocks were analyzed by TEM.

Correlation of cellular dissolution of ZnONMs to cell death using live cell laser scanning confocal microscopy

TT1 cells were cultured on glass-bottomed petri dishes (μ -Dish^{35mm}; ibidi GmbH, UK) and incubated with Hoechst 33342 to stain cell nuclei, and a Zn²⁺-selective indicator (FluoZinTM-3, AM). After addition of PI-containing RPMI-1640, live cell imaging was performed using a Leica SP5 MP inverted confocal microscope (Leica, Germany) with an environmental chamber (37 °C, 5% CO₂).

Statistical analysis

Cell-free dissolution, DLS and ζ -potential experiments were repeated at least three times. Values are shown as mean $\pm$ SD. Statistical analyses were carried out using one-way ANOVA in Origin 9.1. Differences were considered statistically significant at $p < 0.05$.

Toxicity data (WST and LDH) were analyzed using 2-way analysis of variance (ANOVA) with a Post-Hoc (Bonferroni) test to confirm differences between ZnCl₂, S- and L-ZnONWs at different concentrations using GraphPad Prism[®] 5.0. A similar analysis was applied to determine the difference in different treatments ($\pm$ 10% NCS or Curosurf[®]) at different ZnONWs concentrations. In confocal experiments, differences in mean fluorescence intensity (MFI) values of fluorescent signals over time were analyzed using 2-way ANOVA. Differences were considered significant at $**p < 0.01$ and $***p < 0.001$. Values are shown as mean $\pm$ SD.

Results

PS alters ZnONW dissolution kinetics and coats ZnONWs with a phospholipid corona

As-received ZnONWs were thoroughly characterized, as described in detail in the Supplementary Material (Figure S4 and S5), and their physicochemical properties are summarized in Table 1. First, the effects of PS on ZnONW dissolution were examined at different pH values. The main paradigm describing the toxicity of ZnONMs is the accumulation of ZnO inside cells due to cellular uptake of ZnONMs, followed by pH-triggered intracellular dissolution and release of Zn²⁺ ions, which are toxic at elevated levels (Muller et al., 2010). In the absence of PS, ZnONW dissolution was strongly pH-dependent (Figure 1a), in accordance to previous findings (Bian et al., 2011, Illy et al., 2014). At pH 7, both types of ZnONWs tested were relatively stable, with a maximum of 10% of ZnO solubilized. At pH 5, there was an apparent instantaneous and complete dissolution of ZnONWs. No significant differences in dissolution were observed between the two types of ZnONWs tested, at pH 5 or pH 7. These findings supports previous studies, which have reported similar dissolution between bulk- and nano-ZnO particles (Franklin et al., 2007, Mortimer et al., 2010, Li et al., 2011) as well as ZnONMs of different sizes and shapes (Peng et al., 2011).

Next, the effects of PS and 10% NCS on dissolution were examined for S-ZnONWs (Figure 1b, c). At pH 7 (Figure 1b), the presence of DPPC or Curosurf[®] had no effect on the dissolution of S-ZnONWs, as we have also demonstrated for AgNWs (Theodorou et al., 2015a). In contrast, 10% NCS markedly increased ($p < 0.05$) the amounts of Zn²⁺ ions

released at pH 7, which may be due to adsorption of proteins (*e.g.* albumin) from NCS on the ZnONW surface and the formation of a protein corona (Sasidharan et al., 2013). At pH 5 (Figure 1c), DPPC or 10% NCS had no significant impact on the dissolution of S-ZnONWs. A prominent decrease in dissolution was, however, observed in the presence of Curosurf[®]. Instead of immediate dissolution, only 35% of S-ZnONWs had solubilized within 1 hour and 60% after 8 hours. This finding supports our previous work, where Curosurf[®] was more effective than DPPC in delaying the dissolution of AgNWs at pH 5 (Theodorou et al., 2015b). Similar to AgNWs, the reason for a reduced S-ZnONW dissolution could be their coating by phospholipids, which blocks direct contact between the NW surface and the aqueous environment. The same effects of PS and 10% NCS on dissolution were also observed with L-ZnONWs (Supplementary Material, Figure S6)

Since dissolution was the same for S-ZnONWs and L-ZnONWs, their morphological evolution against time and pH was examined by TEM only for S-ZnONWs (Figure 2). After incubation at pH 7 for 1 hour, no noticeable effects were observed in the morphology of the S-ZnONWs (Figure 2a), in agreement with the low dissolution measured at this time point by ICP-OES (Figure 1a). After 24 hours at pH 7, S-ZnONWs had been slightly etched and presented irregular surfaces (Figure 2b). Similar observations were made for S-ZnONWs incubated at pH 7 for 24 hours in the presence of DPPC (Figure 2c), Curosurf[®] (Figure 2d) or 10% NCS (Figure 2e). Since dissolution of S-ZnONWs was very rapid at pH 5 (Figure 1a), their morphology was inspected at very short incubation times. Within 10 seconds at pH 5, in the absence (Figure 2f) or presence of 10% NCS (Figure 2g) or DPPC (Figure 2h), S-ZnONWs had been severely etched. They no longer appeared as continuous wires but had dissolved to smaller individual and irregular particles. The incubation media only contained Cl⁻ ions, therefore the precipitation of insoluble Zn compounds was not expected. The lattice spacing measured from HRTEM images (Figure 2i) was 2.56 ± 0.06 Å, which corresponds to the (002) interplanar spacing of wurtzite ZnO (JCPDS #89-1397). STEM-EDX (Figure 2j, k) revealed only the presence of Zn and O, confirming that the observed structures corresponded to dissolved S-ZnONWs. Similar morphological alterations have been previously reported for ZnONWs in simulated body fluids at pH 7.4 and pH 5.2 (Muller et al., 2010). Zhou *et al.* showed that ZnO wires could dissolve in a range of biofluids. The etching process started at the corners of their hexagonal cross-section, leaving the flat surfaces initially spared and resulting in a rough appearance of the wires (Zhou et al., 2006).

In contrast to the rapid etching of S-ZnONWs at pH 5, incubation of S-ZnONWs at pH 5 in the presence of Curosurf[®] for 10 seconds led to no observable changes to their structure (Supplementary Material, Figure S7). After 24 hours, S-ZnONWs had been considerably etched but still appeared as continuous wires (Figure 2l). These observations support the decrease in S-ZnONW dissolution at pH 5 in the presence of Curosurf[®] measured by ICP-OES (Figure 1c). Imaging at higher magnification revealed the presence of an amorphous layer at the surface of S-ZnONWs (Figure 2m), which was attributed to the coating of S-ZnONWs by phospholipids, as we have recently demonstrated for AgNWs (Theodorou et al., 2015b). The same approach as our previous work was followed to reveal the organization of phospholipids on the surface of S-ZnONWs. After positively staining the samples with uranyl acetate, striations parallel to the long axis of the S-ZnONWs were observed (Figure

2n). Since the hydrophilic head groups of phospholipid molecules produce dark contrast whereas the hydrophobic fatty acid tails produce bright contrast under staining (Hayat, 2000), the outermost surface of the lipid coating of S-ZnONWs, facing the aqueous environment, corresponds to hydrophilic head groups (similar for more than 100 S-ZnONWs inspected). When hydrophilic groups are able to interact with the surrounding aqueous medium, the sorption of organic compounds on nanoparticles facilitates the stability of the nanoparticle dispersion (Chappell et al., 2011). The bilayer thickness, measured by collecting several intensity line profiles (Figure 2o), had an average of 3.9 ± 0.6 nm. This value is the same as the bilayer thickness we have measured on AgNWs incubated with Curosurf[®], and close to the estimated maximum hydrocarbon bilayer thickness of 4.16 nm (Theodorou et al., 2015b). In contrast to our previous work, where phospholipids from various sources (DPPC, Curosurf[®] and *murine* PS) coated AgNWs, no evidence was found that DPPC was able to coat S-ZnONWs (Supplementary Material, Figure S8).

PS reduces the agglomeration of ZnONWs

Even though the use of DLS to accurately measure the dimensions of 1-dimensional NMs is not straightforward, it may be used to estimate agglomeration in NM dispersions. However, since both DPPC and Curosurf[®] are insoluble, they may form lipid vesicles in aqueous solutions. The size of these vesicles is expected to be much lower than the hydrodynamic size of ZnONWs, which is close to 1 μ m. In the size distribution data of S-ZnONWs obtained by DLS, an additional peak/shoulder was observed when S-ZnONWs were incubated with either DPPC or Curosurf[®] (Supplementary Material, Figure S1). The position of this peak was in both cases about 150 nm, and attributed to the formation of lipid vesicles. The peak with the higher intensity was considered as the hydrodynamic size of S-ZnONWs and its position over time is shown in Figure 3a. The hydrodynamic size of S-ZnONWs, in the absence or presence of DPPC, quickly increased within 1 hour of incubation in DI water (Figure 3a). However, for S-ZnONWs incubated with Curosurf[®] or 10% NCS, hydrodynamic size remained relatively constant from 0 up to 48 hours. These results were corroborated with ζ -potential measurements (Figure 3b). In the absence or presence of DPPC, S-ZnONWs had a positive ζ -potential immediately after incubation, which dropped close to 0 within 1 or 4 hours of incubation, respectively. Incubation of S-ZnONWs with either Curosurf[®] or 10% NCS led to a negative ζ -potential, with higher magnitudes in the case of Curosurf[®]. Although in both cases the ζ -potential magnitude slightly decreased over time, it remained lower than -10 mV after 48 hours.

These measurements indicate that, both Curosurf[®] and NCS, but not DPPC, prevent the agglomeration of S-ZnONWs, which may be correlated with the coating of ZnONWs by a phospholipid corona in the presence of Curosurf[®] but not of DPPC (Figure 2m). In the case of NCS, studies have also shown that serum stabilized ZnONPs against aggregation and sedimentation (Hsiao and Huang, 2013). One possible stabilising agent in serum is albumin, the most abundant protein in serum, which may adsorb to the surface of ZnO creating a protein corona (Sasidharan et al., 2013). Albumin carries a negative charge (around -20 elementary charges per molecule) at physiologic pH and may therefore stabilise particles by imparting a net surface charge (Rezwan et al., 2004), as indicated by our ζ -potential measurements.

PS increases ZnONW bioreactivity with TT1 cells

Having examined how the properties of high-aspect ratio ZnONWs change over time at different pH values and in the presence of PS, we proceeded to test their bioreactivity with human alveolar epithelial type 1-like (TT1) cells. Their bioreactivity was assessed by a water soluble tetrazolium salts (WST) assay and lactate dehydrogenase (LDH) release assay, which measure the metabolic activity of cells and necrotic cell death, respectively. To compare with the effects of Zn^{2+} ions, ZnCl_2 was used as a source of soluble ionic zinc. The WST assay demonstrated a time- and concentration-dependent decrease in cell viability following exposure of TT1 cells to the Zn compounds (Figure 4a, b). Compared to the non-treated (NT) control, both S-ZnONWs and L-ZnONWs significantly reduced ($p < 0.01$, $n = 3$) the viability of TT1 cells at doses of 1 $\mu\text{g/mL}$ and above after 4 hours (Figure 4a), whereas the threshold of ZnCl_2 -induced cell death was at 5 $\mu\text{g/mL}$. This threshold, between 1 and 5 mg/mL ZnONWs was a consistent feature of the cell death studies. Thus, after 24 hours, significant cell death ($p < 0.01$, $n = 3$) occurred following exposure to 5 $\mu\text{g/mL}$ and above of all materials tested, the degree and profile of which was similar (cell death plateaued with increasing ZnONW concentration; Figure 4b) and, with the exception of the 1 $\mu\text{g/mL}$ dose, was significantly greater than that observed at 4 hours ($p < 0.01$, $n = 3$). The median lethal doses (LD_{50}) after 24 hours were estimated to be 10.13 $\mu\text{g/mL}$ for ZnCl_2 , 10.90 $\mu\text{g/mL}$ for S-ZnONWs and 11.18 $\mu\text{g/mL}$ for L-ZnONWs, in terms of ionic Zn^{2+} concentration. Therefore, based on mass dosing, both lengths of ZnONWs exhibited similar effects on the viability of TT1 cells, in accordance to the similar dissolution kinetics measured *in vitro* (Figure 1a). Previous studies comparing the cytotoxic effects of spherical and fibrous nanomaterials (George et al., 2012) have noted that, when cytotoxicity is expressed according to a surface area instead of a mass dose metric, differences between different nanoparticles may arise or disappear as a result of correcting for the differences in surface area. However, in the present work, only ZnONWs with two different lengths are compared, and since their radii are the same (Table 1), we expect to obtain the same responses when expressing our data based on a surface area dose metric (as described in detail in the Supplementary Material).

LDH release after 4 hours was not significant ($p > 0.05$, $n = 3$) compared to the NT control for any of the materials at the dose range tested (Figure 4c). Relative to the NT control, exposure to up to 100 $\mu\text{g/mL}$ of ZnCl_2 for 24 hours did not result in significant LDH release (Figure 4d). Addition of ZnCl_2 to the cell culture medium may have caused supersaturation of Zn^{2+} with respect to the zinc carbonate and/or phosphate equilibrium solubility of the medium, leading to the formation of particulates with unknown effects on membrane integrity (Mu et al., 2014). In contrast, both S-ZnONWs and L-ZnONWs caused a similar degree of cellular LDH leakage after 24 hour exposure to doses of 10 $\mu\text{g/mL}$ and above (Figure 4d). Normally, early-stage plasma membrane permeabilization (within 3–6 hours) is associated with necrosis whereas its delayed occurrence is the signature of apoptosis and secondary necrosis at a later stage. Therefore, our findings indicate that ZnONWs induce early-stage programmed TT1 cell death without disrupting the plasma membrane, and, in the absence of macrophage efferocytosis and other cell death clearance mechanisms, induction of late stage cellular necrosis. Using TEM, Muller *et al.* found that human MDMs treated with ZnONWs showed signs of both apoptosis, such as chromatin condensation and mitochondrial pyknosis, as well as necrosis, such as plasma membrane rupture and leaching

of cytoplasmic contents (Muller et al., 2010). This heterogeneity in cellular responses could be due to the cells being exposed to highly variable local concentrations of zinc.

Since both types of ZnONWs and ZnCl₂ had similar effects on the viability of TT1 cells, the effects of PS and 10% NCS were assessed only for S-ZnONWs. Even though the addition of 10% NCS had increased dissolution of ZnONWs at pH 7 (Figure 1b), this had no effect on the viability of TT1 cells compared to that of bare ZnONWs, measured by the WST assay (Figure 4e). In accordance to this finding, Hsiao *et al.* showed that the enhanced extracellular Zn²⁺ release by micro- and nano-ZnO particles in serum-containing tissue culture medium did not contribute as a main factor in the cytotoxicity of the ZnO particles (Hsiao and Huang, 2013). On the other hand, despite the decrease in dissolution at pH 5 (Figure 1c), exposure to S-ZnONWs pre-incubated with Curosurf[®] led to a significantly higher decrease (from 40–50% cell death to 70–80% cell death; $p < 0.001$, $n = 3$) in TT1 viability compared to bare S-ZnONWs, for doses of 5 µg/mL and above (Figure 4f).

PS increases the uptake of S-ZnONWs by TT1 cells

In order to test whether these differences in cell viability arise due to differences in the amounts of S-ZnONWs internalized by the cells, uptake in TT1 cells was quantified by ICP-OES (Figure 5a). After 4 hours of incubation, TT1 cells had taken up similar amounts of ZnCl₂, S-ZnONWs in serum-free medium and S-ZnONWs in medium containing 10% NCS (Figure 5a). In contrast, pre-incubation of the wires with Curosurf[®] led to about a 4-fold increase in intracellular zinc (in serum-free medium; Figure 5a). The percentage of cells positive for S-ZnONW uptake estimated by BFTEM imaging similar indicated a higher degree of internalization for S-ZnONWs pre-incubated with Curosurf[®] compared to bare S-ZnONWs (Figure 5b).

BFTEM imaging of TT1 cells exposed to S-ZnONWs for 4 hours revealed that there were no intact S-ZnONWs intracellularly, in the absence (Figure 5c, d) or presence (Figure 5g, h) of Curosurf[®]. Instead, compared to non-treated control cells (Supplementary Material, Figure S9), electron dense material was observed inside TT1 cells, and TEM-EDX (Figure 5e, i) showed the presence of Zn in the areas around this material, both in the absence (Figure 5e) and presence of Curosurf[®] (Figure 5i). Zn peaks were not detected in TEM-EDX spectra collected from areas in the extracellular space (Supplementary Material, Figure S10). HAADF-STEM imaging, a technique sensitive to local atomic number variations in samples, confirmed the presence of material with high atomic number inside TT1 cells (Figure 5f, j, arrows). This material, resembling observations in cell-free dissolution experiments (Figure 2f), was attributed to remnants of S-ZnONWs following intracellular dissolution. In most of previous studies employing TEM to examine cells exposed to ZnONPs, no particles could be detected intracellularly, possibly due to their rapid dissolution. For instance, within 1 hour exposure of human MDMs to ZnONWs, no intact wires were observed intracellularly, but cells instead contained “ghost” structures of electron dense material (Muller et al., 2010).

PS delays the intracellular dissolution of S-ZnONWs within TT1 cells and the onset of cellular damage

To further investigate the correlation between intracellular ZnONW dissolution and cell death, the cellular distribution and dissolution of S-ZnONWs were dynamically analyzed by live cell laser scanning confocal microscopy. TT1 cells were preloaded with a Zn^{2+} indicator (FluoZinTM-3, AM), a cell-permeant acetoxymethyl (AM) ester that displays green fluorescence upon binding of ionic Zn^{2+} . Propidium iodide (PI), a red fluorescent dye that is membrane impermeable and generally excluded from viable cells, was added to stain the nuclei of necrotic cells. Following introduction of S-ZnONWs, the cells were imaged at regular intervals up to 3 hours (Figure 6). In the absence of Curosurf[®], green fluorescence was observed within 20 min following exposure (Figure 6b), indicating a rise in intracellular Zn^{2+} concentrations. This was confirmed by an increase in the mean fluorescence intensity (MFI) of FluoZinTM-3 (Figure 6k). Within 1 hour, the FluoZinTM-3 MFI had reached its maximum value, followed by a drop in FluoZinTM-3 fluorescence, possibly indicating leakage of the Zn^{2+} -dye complex from the cells. Concomitantly, an increase in PI fluorescence was observed, indicating necrotic cell death. These findings suggest that the toxicity of S-ZnONWs is due to an intracellular increase in ionic Zn^{2+} following uptake and dissolution of the ZnONWs inside cells. However, the LDH assay indicated insignificant release of LDH following a 4h S-ZnONW exposure (Figure 4c); the increase in PI fluorescent, observed here, could be due to secondary necrotic cell death following the rapid release of Zn^{2+} from S-ZnONWs locally. TT1 cells exhibited a heterogeneous response to S-ZnONWs, with some cells dying within 40 minutes (Figure 6c), while others more than 2.5 hours (Figure 6i) after exposure. This heterogeneity may be due to the tendency of S-ZnONWs to agglomerate, leading to exposure of TT1 cells to highly variable concentrations of S-ZnONWs in different areas of the TT1 cell monolayer.

When TT1 cells were exposed to S-ZnONWs pre-incubated with Curosurf[®], no release of Zn^{2+} was observed within cells over the first 3h following addition of S-ZnONWs (Figure 7a–o). This finding indicates that Curosurf[®] may delay the intracellular release of Zn^{2+} from S-ZnONWs, through their coating with a phospholipid corona, as we previously observed in cell-free *in vitro* measurements (Figure 2n). Significant intracellular release of Zn^{2+} from ZnONWs was observed 4h after exposure; however no significant PI fluorescence was measured within the timeframe of the experiment (Figure 8p–q). Since pre-incubation of S-ZnONWs with Curosurf[®] resulted to a higher decrease of TT1 viability at 24 hours compared to bare S-ZnONWs (Figure 5f), these results suggest that, by delaying intracellular dissolution, the phospholipid corona also delayed cellular damage.

Discussion

Since the first interaction of any nanoparticle that reaches the alveoli will be with the pulmonary surfactant (PS), its effects on the physicochemical properties of nanoparticles could have a significant impact on their bioreactivity. In the present work, by performing detailed characterization we were able to provide new insights on the effects of PS on the dissolution and colloidal stability of ZnONWs, as well as their bioreactivity toward TT1 human alveolar epithelial cells.

In summary, *in vitro* adsorption of PS lipids on ZnONWs has been demonstrated for the first time. This lipid corona delayed the kinetics of Zn^{2+} ion release from ZnONWs at acidic pH, by blocking direct contact between the NW surface and the aqueous environment, as previously demonstrated for AgNWs (Theodorou et al., 2015a). TEM imaging indicated that surfactant molecules adsorbed and formed lipid bilayers on the surface of the particles, possibly by virtue of their amphiphilic nature, thus sterically stabilizing the particles (Chappell et al., 2011). DLS and ζ -potential measurements confirmed that PS prevented the agglomeration of ZnONWs, possibly through contributions of both steric and charge stabilization. The absence of similar effects when ZnONWs were incubated with DPPC instead of Curosurf[®] may be due to the more complex composition of Curosurf[®] (Zhang et al., 2011). Apart from DPPC, which is a zwitterionic phospholipid, Curosurf[®] also contains anionic phospholipids, as well as the hydrophobic surfactant proteins SP-B and SP-C (Zhang et al., 2011). Previous findings indicate that ionic surfactant molecules are superior in contributing to both charge and steric stabilization of nanoparticles (Bakshi et al., 2008). Furthermore, SP-B/C are known to act as “fusogen” that promote lipid mixing and increase the adsorption rate of phospholipids (Creuwels et al., 1997). Therefore, our findings suggest that hydrophobic surfactant proteins (SP-B/C) may be present in the ZnONW corona. Previous studies have demonstrated that, upon interaction of NPs with the PS, a lipoprotein corona is formed (Hu et al., 2013). Raesch *et al.* have recently shown that the lipid composition of these coronas was similar, but their protein composition varied markedly depending on the surface properties (hydrophobicity/surface charge) of the NPs investigated (Raesch et al., 2015).

Scanning confocal microscopy imaging of live TT1 cells showed that cell damage correlated with an increase in intracellular ionic Zn^{2+} following uptake and dissolution of ZnONWs inside cells, as previously demonstrated for MDMs (Muller et al., 2010). Moreover, confocal imaging suggested that the coating of ZnONWs by a phospholipid corona slowed down their dissolution kinetics intracellularly, also delaying the onset of cellular damage. The intracellular processing of NP coronas following uptake, and the consequences on NP cytotoxicity, are not well understood. Recent findings suggest that biomolecular coronas are retained by NPs as they are internalized by cells and are trafficked to the lysosomes on the nanoparticle's surface (Wang et al., 2013). In the lysosomes, the coronas may protect the cells from damage induced by the bare nanoparticle, until the biomoecules are enzymatically cleared and the nanoparticle's surface is re-exposed (Wang et al., 2013). For instance, the rate of proteolytic degradation of protein coronas on gold nanoparticles (AuNPs) varied between different proteins, with half times (time required for degradation of 50% of the corona) ranging from around 4 to 70 hours, and this variation correlated with the degree of cell damage (Ma et al., 2015). Similarly, in our experiments, the delayed toxicity of Curosurf[®] pre-incubated ZnONWs may be a result of the time required for the enzymatic degradation of the phospholipid corona, possibly by phospholipases, which are known to be present in lung tissue (Hwang et al., 1996). In type 2 alveolar epithelial cells, the degradation half time of internalized DPPC has been measured at around 2 hours (Fisher and Dodia, 1996). Therefore, further investigation is required in order to fully understand the role of phospholipid coronas on the internalization of NPs, the intracellular fate of the corona and its impact on the accompanying toxicity of NPs.

Despite delaying the onset of cellular damage, pre-incubation of ZnONWs with Curosurf[®] led to uptake of the ZnONWs by a higher percentage of TT1 cells within 24 h; this increase in cellular uptake dominated over their delayed intracellular dissolution and resulted to a higher reduction in cell viability after 24h. The higher extent of intracellular uptake (Figure 5a, b) of S-ZnONWs pre-incubated with Curosurf[®] may be attributed to the formation of a phospholipid corona (Figure 2n). Previous findings demonstrated that human lung lining fluid increased the uptake of polystyrene NPs by human alveolar epithelial cells, regardless of NP size or surface modification (Thorley et al., 2014). Konduru *et al.* suggested that the phospholipid coating on single walled carbon nanotubes represented an “eat me” signal on their surface that facilitated their recognition and internalization by macrophages (Konduru et al., 2009). The exact mechanisms of increased intracellular uptake efficiency after surfactant coating of NPs remain to be investigated in detail. On the one hand, this increase could be due to the reduction in the agglomeration state of ZnONWs following surfactant coating (Figure 3b), as size-dependent uptake of other nanomaterials has been previously observed with TT1 cells (Sweeney et al., 2015). On the other hand, the formation of a lipid shell around the particles (Figure 2n) could have caused an enhanced particle interaction with the cell membrane. Hydrophobic interactions or a membrane fusion process, similar to that described for phospholipid vesicles (Almofti et al., 2003), especially for those having a net negative surface charge, possibly cause this enhancement. Commercially available bovine- and porcine-derived surfactant preparations, for example the one used in this work, although useful clinically and as models of the lung lining fluid (LLF), only contain phospholipids and surfactant proteins B and C, important in maintaining a reduced surface tension. Ideally, to fully appreciate the impact of the LLF on ZnONW uptake, the full protein complement of human LLF (*i.e.* SP-A, SP-B, SP-C and SP-D) should be evaluated. Alone, SP-A and SP-D were previously shown to increase the uptake of magnetite nanoparticles by murine alveolar macrophages (Ruge et al., 2011, Ruge et al., 2012), while Kendall *et al.* showed increased uptake of polystyrene nanoparticles by murine alveolar macrophages and dendritic cells in the presence of SP-D alone (Kendall et al., 2013). In contrast, in our own work, alveolar type II cell secretions reduced AgNW bioreactivity with TT1 cells by reducing their uptake, possibly involving specific binding of SP-A/D to the AgNWs (Sweeney et al., 2015), which we have found induces AgNW agglomeration (Theodorou et al., 2015a). Whether this scenario would be the same for ZnONWs remains to be established.

On the other hand, serum had no significant effect on ZnONW uptake or cell viability, despite also possibly forming a protein corona on the surface of ZnONWs. The exact role of adsorbed protein in NP uptake is not clear, with some studies suggesting that proteins promote NP uptake via receptor-mediated endocytosis while others have demonstrated a reduction or no effect in uptake (Tedja et al., 2012, Hsiao and Huang, 2013). It is likely that differential adsorption of serum protein components by different nanomaterials impacts on the bioreactivity and mode of cellular uptake, if any. Our findings illustrate that the nature of NP corona, and its effects on particle uptake and intracellular fate, have interconnected roles on the interaction between nanoparticles and alveolar epithelial cells, and require further investigation.

Finally, our data indicate that, in the case of ZnONWs, their bioreactivity may not be strongly dependent on their aspect ratio, which did not significantly change their dissolution kinetics or toxicity against TT1 cells. For other types of fibrous nanomaterials (*e.g.* carbon nanotubes, AgNWs), frustrated phagocytosis has been stated as a key determinant for the onset of inflammatory reactions. For AgNWs, the onset of frustrated phagocytosis *in vivo* was determined at a length >10 μm , while most of the shorter AgNWs (<5 μm) were fully internalised by pleural macrophage cells, following intra-pleural instillation (Schinwald and Donaldson, 2012). Indeed, we originally planned to study ZnONWs 5–10 μm in length. After many attempts to grow ZnONWs to this length (by electrochemical deposition) and upscale the synthesis, we were unsuccessful. This suggests that at this stage, mass production of longer, respirable ZnONWs is unlikely to be an occupational hazard. Commercial ZnONWs longer than 3 μm were not available, while their actual dimensions measured by TEM were different from the nominal dimensions stated by the manufacturer (90 nm $\times$ 1 μm for S-ZnONWs and 300 nm $\times$ 4–5 μm for L-ZnONWs). Previous studies have also shown that the toxicity of spherical and rod-shaped ZnONPs (10–200 nm) to mouse neuronal stem cells was independent of particle shape and size, and similar to ZnCl₂ (Deng et al., 2009). For both ZnONW lengths tested here, the estimated LD₅₀ values were close to LD₅₀ doses previously measured for human MDMs exposed to ZnONWs (Muller et al., 2010).

Conclusions

In conclusion, our findings show that the PS can significantly alter the dissolution kinetics, aggregation state and surface chemistry of ZnONWs, with important consequences on how they are internalized and processed by the underlying epithelial cells. This work highlights that combinations of spatially resolved static and dynamic techniques are required to develop a holistic understanding of the parameters that govern ZnONW bioreactivity at the point of exposure and to accurately predict their risks on human health and the environment. More generally, our observations illustrate that the role of PS is central in understanding interactions at the bio-nano interface of the alveoli, and their impact on subsequent epithelial–endothelial nanoparticle translocation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1. PS reduces the dissolution kinetics of S-ZnONWs at lysosomal pH

(a) Inductively coupled plasma optical emission spectroscopy (ICP-OES) analysis of the amount of free Zn^{2+} ions released from S-ZnONWs and L-ZnONWs incubated in normal saline (NS) solutions at pH 7 and pH 5, for 0 up to 48 hours. (b, c) ICP-OES analysis of the amount of free Zn^{2+} ions released from S-ZnONWs incubated in NS at pH7 (b) or pH5 (c), in the absence or presence of DPPC, Curosurf[®] or 10% NCS. Each experiment was repeated 3 times (n=3) and data are presented as mean $\pm$ SD.

Figure 2. PS reduces the etching of S-ZnONWs at lysosomal pH and forms a phospholipid corona

(a–e) BFTEM images of S-ZnONWs incubated in NS at pH7 for 1 hour (a) or 24 hours (b–e), in the absence (a, b) or presence of DPPC (c), Curosurf[®] (d) or 10% NCS (e). (f–h) BFTEM images of S-ZnONWs incubated in NS at pH5 for 10 seconds, in the absence (f) or presence of 10% NCS (g) or DPPC (h). (i) HRTEM image of the area marked 1 in (h). (j) High angle annular dark field scanning transmission electron microscopy (HAADF-STEM) image of S-ZnONWs incubated in NS at pH5 for 10 seconds (k) STEM-EDX spectrum collected from the area marked 2 in (j). (l–n) BFTEM images of S-ZnONWs incubated in NS at pH5 in the presence of Curosurf[®] for 24 hours. (m) is the magnification of the area squared in (l). In (n) samples were positively stained with uranyl acetate (UA) to enhance phospholipid contrast and shows multiple phospholipid bilayers formed on the surface of S-ZnONWs. (o) Intensity line profile collected from the line marked 3 in (n).

Figure 3. PS decreases the agglomeration of S-ZnONWs and imparts a negative surface charge
 (a) Hydrodynamic size and (b) ζ -potential of S-ZnONWs incubated in DI water, in the absence or presence of DPPC, Curosurf or 10 % NCS. Each experiment was repeated 3 times ($n=3$) and data are presented as mean $\pm$ SD.

Figure 4. PS increases the bioreactivity of S-ZnONWs with type 1-like (TT1) human alveolar epithelial cells

(a, b) Cell viability (WST assay; n=3, **p<0.01, ***p<0.001) and (c, d) LDH release (n=3, **p<0.01, ***p<0.001) following treatment of TT1 cells with S-ZnONWs and L-ZnONWs for 4 hours (a, c) and 24 hours (b, d). ZnCl₂ was used as a control for free Zn²⁺ ions. (e, f) Cell viability (WST assay; n=3, **p<0.01, ***p<0.001) following treatment of TT1 cells with S-ZnONWs for 24 hours, in the absence or presence of 10% NCS (e) or Curosurf® (f).

Figure 5. PS increases the S-ZnONW uptake by human alveolar epithelial cells

(a) ICP-OES quantification of the amount of intracellular Zn²⁺ in TT1 cells, following treatment for 4 hours with ZnCl₂ or S-ZnONWs, in the absence or presence of 10% NCS or Curosurf[®]. Experiments were repeated 3 times (n=3) and data are presented as mean ± SD. (b) BFTEM quantification of intracellular Zn in TT1 cells treated for 4 hours with S-ZnONWs, in the absence or presence of Curosurf[®]. Experiments were repeated 3 times (n=3) and data are presented as mean ± SD. BFTEM images (c, d, g, h) of TT1 cells exposed to S-ZnONWs in the absence (c, d) or presence (g, h) of Curosurf[®] (n=3). (d) and (h) are magnifications of the areas squared in (c) and (g) respectively. (e, i) TEM-EDX spectra

collected from the areas circled in (d) and (h) respectively. (f, j) HAADF-STEM images of TT1 cells exposed to S-ZnONWs in the absence (f) or presence (j) of Curosurf[®] (n=3). (C: cytoplasm; ES: extracellular space)

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Figure 6. Intracellular dissolution of S-ZnONWs within live TT1 cells correlates to cell viability (a–j) Live TT1 cells were incubated with S-ZnONWs at 37°C with 5% CO₂ and imaged by laser scanning confocal microscopy every 20 minutes (a–j) up to 3h. Cells were stained with Hoechst 33342 (blue, nuclei) and FluoZin™-3, AM (green, Zn²⁺) prior to exposure to S-ZnONWs in PI-containing (red, necrotic cells) serum free RPMI cell culture medium. (k) The dynamic intracellular dissolution of S-ZnONWs (green line) and their effect on cell viability (red line; PI), were quantified by measuring the mean fluorescent intensity (MFI) of FluoZin™-3, AM (l; green in a–j) and PI (m; red in a–j) against time in six cells from one single experiment. Region of interests (ROI 1–6, squared in l, m) indicate the areas where the fluorescent signals were measured and plotted in k. (n) Three experiments were performed with a total of 21 cells observed, and the signals of Zn²⁺ and PI were plotted against the time in comparison to the background signals obtained from non-treated cells (NT-Zn²⁺ and NT-PI; n=3 with a total of 21 cells observed, ***p<0.001).

Figure 7. PS delays intracellular dissolution of Curosurf[®]-preincubated S-ZnONWs within live TT1 cells, and the onset of cell damage

(a–o) Live TT1 cells were incubated with S-ZnONWs pre-incubated with Curosurf[®] and imaged by laser scanning confocal microscopy every 20 minutes up to 5h. Cells were stained with Hoechst 33342 (blue, nuclei) and FluoZin[™]-3, AM (green, Zn²⁺) prior to exposure to S-ZnONWs in PI-containing (red, necrotic cells) serum free RPMI cell culture medium. (p) There was no significant Zn²⁺ release from the Curosurf[®]-preincubated S-ZnONWs (green line) and no necrotic cell death (red line) was observed within 3 hours of exposure. A significant release of Zn²⁺ ($p < 0.001$; $n = 3$ with a total of 21 cells observed) was observed after 4h of exposure (green line in p, yellow arrows in q indicate areas of Zn²⁺ release), with no significant necrotic cell death (red line in p). S-ZnONWs, imaged in reflectance mode (magenta in q, white arrows) remained in the system close to areas of Zn²⁺ release.

Summary of physicochemical properties of “short” (S-ZnONWS) and “long” (L-ZnONWS) ZnO nanowires.

Table 1

Material	Diameter (nm) (TEM)	Length (μm) (TEM)	DI water (pH 6.4)			RPMI-1640 (pH 7.2)		
			Hydrodynamic size (nm)	PDI	ζ-potential (mV)	Hydrodynamic size (nm)	PDI	ζ-potential (mV)
S-ZnONWS	28 ± 15	1.3 ± 1.1	815 ± 104	0.35	16.0 ± 0.9	1164 ± 130	0.44	-10.4 ± 0.6
L-ZnONWS	27 ± 16	3.3 ± 2.9	957 ± 132	0.33	11.9 ± 0.3	1319 ± 190	0.44	-10.3 ± 0.8