

The Influence Of Nanoparticle Charge And Hydrophobicity Upon Translocation Across Respiratory Epithelial Cell Layers

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Summary

It is difficult to study the influence of nanoparticle (NP) physicochemical properties upon cell uptake and translocation because NP charge and size is often modified upon suspension within biological fluids. The aim this study was to use non-aggregating, well characterised polyvinyl alcohol (PVA) NPs to investigate how particle charge and hydrophobicity influence uptake and translocation across Calu-3 cell lines. Several grades of PVA were synthesised, characterised and labelled covalently with carboxyfluorescein (CF). The labelled PVA was used to fabricate NP and these particles were suspended in physiological buffer, characterised and applied to the apical surface of Calu-3 cell lines. Up to 11% of the applied particle dose was internalised by the Calu-3 cells, but the maximum particle dose which translocated this confluent barrier was 1.3%.

Introduction

When employed as drug carriers the large surface area of nanoparticles (NP) can improve drug dissolution or enhance controlled release compared to micron sized drug carriers (Emerich and Thanos, 2007). However, a strong propensity to aggregate (Vauthier *et al.*, 2008), the generation of reactive oxygen species (Rodoslav *et al.*, 2003) and long-term accumulation in the systemic circulation (Bazile *et al.*, 1992) are potential problems which must all be avoided if NP drug carriers are to be used clinically. NP toxicity and aggregation are material specific effects and it can be argued that if the correct material is employed to form the NP these problems could be avoided. However, the development of a versatile, biocompatible, non aggregating NP in the dry state is not trivial and the technological challenge of producing such a NP makes the industrial scale manufacture of this material too time consuming and costly for clinical applications.

Surprisingly few studies have been published that report the fate of respirable NP after deposition upon the respiratory epithelium. The data that is available relates to either model NP such as gold or polystyrene, or those NP that are associated with incidental human exposure such as titanium. Analysis of this literature reveals that, despite using well defined non-soluble particles that are much easier to track compared to those typically employed to administer drugs, there is inconsistency in the nature and extent of NP uptake and translocation reported. Whilst both polystyrene 240 nm NP and gold NP with a size of < 100 nm have been shown to translocate the respiratory epithelium when administered to rats there are several reports that show similar sized NP do not translocate the respiratory epithelium (Kato *et al.*, 2003; Bajanowski *et al.*, 1998; Kreyling *et al.*, 2002; König *et al.*, 1993). In order to employ NP to deliver drugs to the respiratory epithelium safely, it is necessary to understand the fate of these particles in the lung. Therefore the aim of this study was to produce pharmaceutically-relevant NP that do not aggregate in physiological salt solution to investigate the capacity of particles with different physicochemical characteristics to be taken up and translocate across airway epithelial cell layers *in vitro*. Several grades of polyvinyl alcohol (PVA) were produced by altering the percentage hydrolysis of the polymer and these were used to manufacture NP. On the basis of NP charge, size, hydrophobicity and labelling efficiency three grades of PVA were selected for fluorescent-labelling to enable the production of NP that could be tracked. Finally, NP formed from the labelled polymers were evaluated for their uptake and translocation in Calu-3 cell layers.

Materials and Methods

Three different PVA grades (40%, 50% and 60% hydrolysed) were produced using high molecular weight (HMW) and low molecular weight (LMW) polymer assuming a stoichiometric 1:1 reaction between the vinyl acetate monomer and the NaOH using a method identical to that published previously (Chana *et al.*, 2008). A selection of these polymers were labelled using a mixture of triphenylphosphine (0.16 mM), PVA (0.0058 mM), diisopropyl azodicarboxylate (0.15 mM) and carboxyfluorescein (CF; 0.13 mM) in dry DMF (10 mL) which was stirred overnight at room temperature. PVA NP were prepared using a nanoprecipitation method (Chana *et al.*, 2008). Briefly,

an aqueous dispersing medium consisting of 4 ml of a 10% (w/v) PVA 80% hydrolysed solution, and 26 ml of a 1.54% (w/v) PVP K15 solution, was homogenised for 30 min (L4RT homogenizer, Silverson, East Longmeadow, MA, USA). The diffusing phase (3 ml) containing 1% (w/v) synthesised PVA (labelled with CF) in methanol was added drop-wise to the dispersing medium using a syringe pump set at 8 mm/min, from a 5 ml syringe (Razel Scientific Instruments Inc.). The particle size and zeta potential of the commercial polystyrene (PS) and manufactured PVA NP was measured using dynamic light scattering (ZetaPlus, Brookhaven Instruments Corporation, US) at 25°C, using a set solid weight concentration of 0.01% (w/v, dilution in HBSS, the medium that was to be employed in the cell culture studies).

Cell layers for the permeability experiments were grown according to the methods of Grainger and co-workers (2006). Briefly, cells were seeded at a density of 10^5 cells/cm² on Transwell® cell culture supports (polyester membrane, 0.4 µm pore size) with 500 µl culture medium in the apical chamber and 1500 µl in the basolateral chamber. After 2 d, the medium was removed from both chambers of the Transwell® and replaced only in the basolateral chamber of the Transwell® support to provide air interface culture conditions. Cell layers were used for experiments between 9-14 days in culture. At t=0, 510 µl of the NP suspension was added to the apical surface of confluent Calu-3 cell layers (TER > 300 Ω cm²). Solid weight concentrations of the NP were used to achieve a particle number of 9.77×10^{10} per ml. The translocation experiments were performed at 37°C using an atmosphere of 5% CO₂ in a humidified incubator under stirring provided by an orbital shaker operated at 50 rpm. At 14 h, the entire donor and receiver fluid were removed and the amount of NP in these vehicles determined using a fluorescence assay. To measure the NP cell uptake the donor and receiver chambers were filled with a 10% SDS solution and cells left overnight to lyse. The next day the amount of NP in the solubilised cell homogenate was determined using the fluorescence assay.

Results and Discussion

Although it would have been ideal to select a wide range of particle hydrophobicity for the subsequent cell uptake and translocation studies, the CF-labelling efficiency was greatest with the most hydrophobic grades due to the reaction and purification conditions employed. Therefore the PVA grades selected to generate the CF labelled NP were PVA HMW 21% hydrolysed (PVA₂₁), PVA LMW 30% hydrolysed (PVA₃₀), and PVA HMW 34% (PVA₃₄). These grades of PVA were selected as they were labelled efficiently and they generated NP similar in size whilst still different in terms of hydrophobicity. PVA₃₄ and PVA₃₀ polymers were labelled most efficiently (table 1).

Table 1. The physicochemical properties of different % hydrolysis carboxyfluorescein labelled poly(vinyl alcohol) (PVA) nanoparticles suspended in HBSS at a 0.05 mg/ml. T represents time. All measurements that include an estimation of error were performed three times and ± one standard deviation is displayed.

<i>Nanoparticle</i>	<i>Labelling</i> (<i>w/w</i> %)	<i>Size, T=0 h</i> (<i>nm</i>)	<i>Size, T=24 h</i> (<i>nm</i>)	<i>Charge (mV)</i>
PVA ₃₀	0.039	183 ± 11	187 ± 5	13 ± 3
PVA ₃₄	0.057	186 ± 2	200 ± 6	19 ± 6
PVA ₂₁	0.017	231 ± 9	219 ± 1	3 ± 3
Polystyrene (PS)	-	293 ± 29	306 ± 19	-13 ± 3

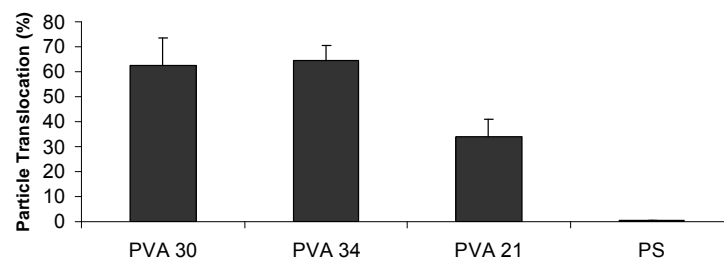
The lack of characterisation of particles in the experimental media in studies reported to date limits their usefulness in evaluating the fundamental interactions of NP with cells. One of the problems that prevents in situ particle size measurement is the inability to measure the particle size of NP in concentrated suspensions using commercially available dynamic light scattering equipment. In this study, the issues caused by a high particle concentration, which induce multiple back scattering effects, were addressed by making all in situ measurements at a single concentration of 0.01% w/v. Whilst the absolute magnitude of the size and charge may change as a result of particle concentration, it was assumed that the relative differences would remain constant. PVA, an uncharged polymer, displayed a positively charged surface when formed into a NP ranging from +3 to +20 mV (table 1). In contrast, polystyrene, which contains surface OH functional groups, exhibited a negative charge when the NP were evaluated in Hank's balanced salt solution (HBSS; table 1). The charge on the PVA particles was assumed to be a consequence of electrolyte adsorption induced by hydration when dispersed in HBSS, but like the PS NP it did not lead to any gross particle size

changes over the 24 h of suspension in the HBSS (table 1). There was not a statistically significant increase ($p > 0.05$, ANOVA with post hoc tukey test) in particle size for the PVA₃₀ or the PS NP at $t = 0$ compared to $t = 24$ h. The PVA₃₄ and PVA₂₁ NP increased in size by $< 7\%$ over the 24 h.

The experimental methodology used in this work was designed to control the potentially confounding issues which arise when studying the kinetics of particulates and allow a direct comparison of NP transport rate in cell layers. In this experiment the NP were shown to be physically stable (table 1), 3 of the 4 NP (PVA₃₀; PVA₃₄; PVA₂₁) were shown to permeate the Transwell® support freely, all the NP suspensions applied to the cells were equivalent in terms of particle number (9.77×10^{10} particles per ml), mass balance was determined by removal of the whole liquid volume and a robust and sensitive assay (developed in previous work, Gul *et al.*, 2008) was used to quantify the NP concentration.

The NP assessed in the uptake and translocation studies were of similar size i.e. 200-300 nm, but the particles were made from materials that displayed different physicochemical properties. In terms of hydrophobicity, the NP were ranked PVA₃₄ $<$ PVA₃₀ $<$ PVA₂₁ $<$ PS according to the properties of the materials from which they were fabricated. The most hydrophilic particles in this set displayed the most extensive transport through the cell-free filter at $63 \pm 11\%$ (PVA₃₀) and $64 \pm 6\%$ (PVA₃₄). In addition, these particles gave the highest recovery for the experiment to study translocation across the epithelial cell layer at 93% and 91% for the PVA₃₀ and the PVA₃₄ NP, respectively. The PS NP did not pass the Transwell® support (meaning that translocation could not be measured in this system) and $< 40\%$ of the PVA₂₁ NP managed to cross the support. The differences in the NP transport through the Transwell support suggests that the study of NP translocation across the cell-free membrane should always be performed even if the NP are of similar size and disaggregated in the dispersion medium.

(a.)



(b.)

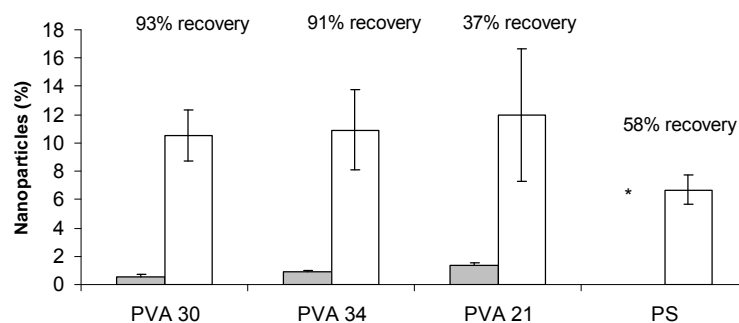


Figure 1. Nanoparticle (NP) translocation through a cell-free Transwell support (a) and particle uptake (white) and translocation (grey) across Calu-3 cell layers (b). * indicates that NP translocation was not possible as the particles did not pass across the support. In the description of the NP the number represents the actual % hydrolysis, PS 250 NC represents polystyrene 250 nm NP.

The PVA NP were internalised most extensively at ca 11 % (figure 1). The uptake of PVA NP by the Calu-3 cell line was independent of surface charge or particle hydrophobicity, but it was significantly higher ($p < 0.05$, t-test) compared to the negatively charged PS NP (7 ± 1 %). The measurement of

uptake may have included particles adsorbed to cell surfaces, but the excellent physical stability of all the particles in the application vehicle, HBSS, demonstrates a favourable NP-vehicle interaction, making this solvent efficient at removing adsorbed particles during the washing stages of the translocation study protocol. In addition, confocal images confirmed qualitatively that particles were internalised within the cells (data not shown).

Conclusions

Understanding how and why NP are transported across airway epithelial cells is critical in order to be able to assess the potential systemic exposure of new respirable materials and to facilitate the rational design of effective inhaled NP drug carriers. The data presented in this study illustrates that in order to discover how the properties of NP influence cell uptake, characterisation of the particles must be performed in the cell lining fluid as the particles can acquire a different charge and display a different particle size compared to the original manufacturer particle specification. NP with a diameter of up to 300 nm were shown to be internalised by Calu-3 cell lines cultured at the air-liquid interface. The preferential internalisation of neutral or +ve charged NP was consistent with a caveolae-mediated endocytotic mechanism of internalisation which has been reported for similar sized particles in previous work. The low levels of NP translocation observed suggest that inhaled NP drug carriers would release their drug pay load at luminal or intra-epithelial locations providing effective delivery to lung epithelium.

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