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Quantitative assessment of nanoparticle surface hydrophobicity and its influence on pulmonary biocompatibility

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ABSTRACT

To date, the role of nanoparticle surface hydrophobicity has not been investigated quantitatively in relation to pulmonary biocompatibility. A panel of nanoparticles spanning three different biomaterial types, pegylated lipid nanocapsules, polyvinyl acetate (PVAc) and polystyrene nanoparticles, were characterized for size, surface charge, and stability in biofluids. Surface hydrophobicity of five nanoparticles (50–150 nm) was quantified using hydrophobic interaction chromatography (HIC) and classified using a purpose-developed hydrophobicity scale: the HIC index, range from 0.00 (hydrophilic) to 1.00 (hydrophobic). This enabled the relationship between the nanomaterial HIC index value and acute lung inflammation after pulmonary administration to mice to be investigated. The nanomaterials with low HIC index values (between 0.50 and 0.64) elicited little or no inflammation at low (22 cm^2) or high (220 cm^2) nanoparticle surface area doses per animal, whereas equivalent surface area doses of the two nanoparticles with high HIC index values (0.88–0.96) induced neutrophil infiltration, elevation of pro-inflammatory cytokines and adverse histopathology findings. In summary, a HIC index is reported that provides a versatile, discriminatory, and widely available measure of nanoparticle surface hydrophobicity. The avoidance of high (HIC index > ~0.8) surface hydrophobicity appears to be important for the design of safe nanomedicines for inhalation therapy.

1. Introduction

Inhalation of drug-containing nanoscale carriers has been heralded as an important strategy for the local or systemic delivery of therapeutic agents to or via the lungs [1–5]. Inhaled nanocarriers have the potential to control drug release, prolong lung retention, target drug to specific sites in the lungs and reduce off-target side effects [6,7]. For successful development as inhaled medicines, however, careful biomaterial design is required to avoid lung toxicity. Paradigms are emerging to link specific physicochemical properties of nanoparticles with acute lung toxicity (e.g. tissue damage, cellular influx or cytokine release) or long term adverse effects such as chronic inflammation, fibrosis, or lung cancer [8–12]. For example, there is a large body of evidence relating respiratory toxicity to nanoparticle properties such as chemically reactive surfaces, including those which generate reactive oxygen species [13], highly cationic or anionic surfaces [14–16], high particle aspect ratio [17], excessive surface area exposure of inert nanomaterials [18,19], and biopersistence in the lungs [20]. Nanomedicine design strategies which avoid or mitigate these risk factors have been shown to result in enhanced biocompatibility profiles *in vivo* [15,21–23].

Surface hydrophobicity is often cited as an additional risk factor for nanoparticle toxicity [20,24,25], but is seldom, if ever, characterized during nanoparticle profiling, which typically concentrates on particle size, shape, zeta potential, crystallinity, colloidal stability, surface reactivity, and solubility/degradability [12]. The rare studies that have investigated the relationship between nanoparticle surface hydrophobicity and *in vivo* respiratory toxicity [15], appear to indicate a relationship, but have lacked a quantitative metric for hydrophobicity. Several analytical methods are available to quantify nanoparticle hydrophobicity, including small molecule adsorption assays (i.e. dyes, hydrophobic compounds, water) [26–28] and hydrophobic interaction chromatography (HIC) [29], each of which has advantages and limitations.

Methods that measure small molecule adsorption to the particle surface provide a highly information-rich characterisation of the molecular interaction forces at the particle surface. For example, Xia et al. [24,25] studied the adsorption profiles of 28 different small molecule probes to the surface of multi-walled carbon nanotubes and their carboxylated derivatives using solid phase microextraction gas chromatography/

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mass spectroscopy. Numerical data representing five descriptors (hydrophobicity, hydrogen bonding, polarity, polarizability, and lone-pair electrons) were combined into a single value, the biological surface adsorption index (BSAI) score, which was proposed for use in modeling quantitative structure–activity relationships. Although this approach is excellent for in-depth analysis of nanoparticle surfaces, its implementation as a routine screening method is limited by the complexity and prolonged duration of analysis, as well as the requirement of specialized analytical equipment.

The aims of this study were to develop HIC as a quantitative method for measuring nanoparticle surface hydrophobicity and investigate the influence of nanoparticle surface hydrophobicity on pulmonary biocompatibility. An HIC index value was developed to provide a quantitative descriptor of nanoparticle surface hydrophobicity and used to score five nanoparticle systems based on three different biomaterial types. Polystyrene (PS) nanoparticles were used as a high hydrophobicity reference nanomaterial reported to elicit an acute inflammatory response in the lung at elevated exposure doses [18,19,22]. Nanoparticles fabricated from two different grades of polyvinyl acetate (PVAc) were included as representative of polymeric systems previously investigated to serve as inert reference particles in inhalation toxicology studies [30] and are also found in some aerosolized consumer products, including hairsprays [31]. Two lipid nanocapsule (LNC) formulations (nanocarriers characterized by a liquid triglyceride core and a solidified phospholipid shell containing pegylated 12-hydroxystearate) were included in the study to exemplify promising nanomedicine formulations for pulmonary delivery of poorly soluble compounds [32–36].

The nanoparticle systems included in the study were designed such that they would display a range of surface hydrophobicities. All the nanoparticles were chemically inert, amorphous, spherical with diameters of either 50 or 150 nm and had a negligible surface charge. The hypothesis of the study was that increasing nanoparticle surface hydrophobicity, as measured by HIC, would correlate with acute respiratory toxicity when administered to the lungs of mice.

2. Materials and methods

Two molecular weight grades of PVAc, high (48 kDa) and low (12.8 kDa), were purchased from Sigma-Aldrich (Dorset, UK). Polyvinyl alcohol (PVA; 8–12 kDa) was purchased from Sigma-Aldrich (Dorset, UK). Labrafac® Lipophile WL1349 was obtained from Gattefosse (Saint-Priest, France), Solutol® HS15 was acquired from BASF (Ludwigshafen, Germany) and Lipoid® S75-3, from Lipoid GmbH (Ludwigshafen, Germany). Non-modified polystyrene nanoparticles with a diameter of 50 nm (2.62% m/v) were used as a reference material and were purchased from Polysciences (Eppelheim, Germany). All other materials were of analytical grade.

2.1. Nanoparticle fabrication

2.1.1. PVAc nanoparticles

To produce differential degrees of hydroxylation, the PVAc polymers were modified by direct saponification according to the method described by Chana et al. [37] producing a PVAc polymer with 17 mol% hydroxyl groups and 83 mol% residual acetate groups (PVAc80%) from the high molecular weight precursor and a modified PVAc polymer with 34 mol% hydroxyl groups and 66 mol% residual acetate groups (PVAc60%) from the low molecular weight polymer. Polymer purity and degree of hydrolysis were verified by NMR analysis prior to use [37]. PVAc60% nanoparticles were prepared by injecting a solution of 5% w/v PVAc60% polymer dissolved in 2:1 methanol:water into a 0.33% w/v aqueous PVA solution, while stirring at 3500 rpm using a Silverson L4 homogenizer (Silverson Machines Ltd., Waterside, UK). PVAc80% nanoparticles were prepared by injecting a solution of 1% w/v PVAc80% polymer dissolved in 2:1 methanol:water into a 0.33% w/v aqueous PVA solution, while stirring at 3500 rpm. Following 30 min constant stirring at 4000 rpm and solvent evaporation overnight (~100 rpm), the nanosuspensions were dialyzed against water (72 h) to remove excess PVA and subsequently concentrated to the desired final concentration using ultrafiltration centrifuge tubes (100 kDa MWCO; Millipore, Watford, UK). Residual PVA was quantified colorimetrically using a method adapted from Sahoo et al. [38].

2.1.2. Lipid nanocapsules

LNCs were manufactured using a phase-inversion temperature method [39]. LNCs with 50 nm diameters (LNC50) were prepared by generating a coarse emulsion of 17% w/w Labrafac® Lipophile WL1349, 17% w/w Solutol® HS15, and 1.75% w/w Lipoid® S75-3 in a 3% w/w NaCl solution. This emulsion was then submitted to repeated heating cooling cycles (85°, 60°, 85°, 60°, 85 °C) before adjusting to 72 °C. At this point the mixture was quenched in a 2-fold volume of ice water and stirred at room temperature for 5–10 min. LNC with 150 nm diameters (LNC150) were prepared and purified using a similar method, but the relative concentrations of components were: 25% w/w Labrafac® WL1349, 8.5% w/w Solutol® HS15, 1.5% w/w Lipoid® S75-3. In the final step of manufacture, the nascent LNC150 nanoparticles were diluted with 2.5 volumes of ice cold water.

Excess stabilizer (Solutol® HS15) was removed from all suspensions by dialysis (72 h) against water containing BioBeads® (BioRad, Hertfordshire, UK) and subsequent concentration using ultrafiltration centrifuge tubes (Millipore, UK; 100 kDa MWCO). Residual Solutol® HS15 was determined colorimetrically [40]. Briefly, following LNC purification by ultrafiltration (as described above), a 50 μL aliquot of the ultrafiltrate containing Solutol® HS15 was added to equal volumes (600 μL) of chloroform and an aqueous solution of ammonium ferrothiocyanate (16.2 g L^{-1} anhydrous ferric chloride; 30.4 g L^{-1} ammonium thiocyanate). The biphasic mixture was incubated under gentle stirring for 30 min at room temperature. The bottom chloroform layer was assayed spectrophotometrically at $\lambda = 510$ nm and the Solutol® HS15 content determined from a calibration curve.

2.2. Nanoparticle characterization

2.2.1. Nanoparticle size and zeta potential

Particle size and zeta potential were determined using a Zetasizer Nano ZS (Malvern, Worcestershire, UK). Size was measured at 25 °C in purified water and 5% dextrose over four weeks to assess storage stability. Stability after aerosolization was assessed by aerosolizing 25 μL nanoparticles suspended in 5% m/v dextrose with a Microsprayer® device (Penn-Century Inc.; Wyndmoor, PA, USA) into 1 mL purified water at 25 °C. Size stability in biological medium was assessed by aerosolizing 25 μL nanoparticles suspended in 5% m/v dextrose into HBSS containing 10% v/v FBS at 37 °C and measuring particle size at t = 0, 0.17, 4 and 24 h. Measurements were taken at a scattering angle of 173°. Refractive indices and viscosity values were adjusted for each temperature and medium used. While all nanoparticle suspensions were size-stable when stored in purified water at 4 °C, fresh batches were prepared for each *in vivo* experiment. Zeta potential measurements were performed at 25 °C with all suspensions diluted in 6.3 mM NaCl.

2.2.2. Hydrophobic interaction chromatography

Surface hydrophobicity of nanoparticle suspensions (n = 3 individual batches) was assessed using HIC [29]. Briefly, nanoparticle suspensions were prepared in PBS (~1 mg mL^{-1}) and 250 μL eluted through three different HiTrap™ substituted sepharose hydrophobic interaction columns: Butyl FF, Phenyl FF (high substitution) and Octyl FF (GE Healthcare Life Sciences, Little Chalfont, UK). The eluent was collected in 1 mL fractions and analyzed for particle content via turbidity measurement (Lambda 35; Perkin-Elmer, Cambridge, UK; $\lambda = 450$ nm). Particles were subsequently eluted from the column using 0.1% Triton X-100. Absorbance values were plotted against elution volumes and two area under the curve (AUC) values were calculated using Origin™