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Influence of amine-modified poly(vinyl alcohol)s on vibrating-membrane nebulizer performance and lung toxicity

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ABSTRACT

A suitable aerosol droplet size and formulation output rate is essential for the therapy of lung diseases under application of nebulizers. The current study investigated the potential of amine-modified poly(vinyl alcohol)s as excipients for inhalation delivery.
A change of conductivity (effective at <0.1 mg/ml) and viscosity (effective at >0.1 mg/ml) of samples that were supplemented with charge-modified polymers had a significant influence on the generated droplet size (shift from ~8 to ~4 μm) and formulation throughput rate (shift from ~0.2 to ~1.0 g/min), where polymers with a higher amine density (and molecular weight) showed an elevated activity. Biocompatibility assessment of polymers in A549 cells and an isolated lung model resulted in cell lysis and lung edema formation dependent on the type (degree of amine substitution) and dose of polymer applied.
Suitable compositions and concentrations of amine-modified poly(vinyl alcohol)s were identified with respect to an optimized nebulizer performance and acceptable biocompatibility. Charge-modified polymers represent novel excipients with potential to improve inhalation therapy.

1. Introduction

Nebulization is an adequate means to generate micron-sized medicament mists suitable for inhalation therapy of airway diseases (Dolovich and Dhand, 2011; Martin and Finlay, 2014). Among the diverse nebulizers that have been described for this purpose, vibrating-membrane technology seems to replace "conventional" systems (i.e., driven by jets and ultrasound), which display significant disadvantages such as medicament concentration variation, high residual volumes and effects on formulation integrity (Bohr and Beck-Broichsitter, 2015). The Aeroneb® Pro and eFlow®rapid represent the most advanced devices currently available utilizing piezoelectric-actuated metal plates containing tapered micro-scale perforations for formulation atomization (Waldrep and Dhand, 2008).

Defined nebulizer performance is of significant interest for inhalation therapy (Bohr and Beck-Broichsitter, 2015; Martin and Finlay, 2014). On one hand, it is well documented, that the size of particles/droplets administered to the respiratory tract should be preferably between 1 and 5 μm to enable an efficient lung deposition pattern (Hofmann, 2011). On the other hand, a high formulation throughput rate is generally preferred in order to reduce the burden of therapy and thus, to improve the quality of life of patients (Gessler et al., 2011). Interestingly, the process of vibrating-membrane nebulization interferes with physicochemical formulation parameters, which can be adjusted by application of particular excipients (Beck-Broichsitter et al., 2014b; Beck-Broichsitter et al., 2014c; Beck-Broichsitter et al., 2014d; Beck-Broichsitter et al., 2014e; Najlah et al., 2013). Therefore, the identification of additives rendering multiple physicochemical parameters of formulations such as (synthetic) macromolecules seems to be of special interest (Beck-Broichsitter et al., 2014c; Bohr and Beck-Broichsitter, 2015; Elphick et al., 2015).

Currently, positively-charged polymers are popular excipients for lung delivery, due to the capability to complex nucleic acids (i.e., gene delivery) (Beck-Broichsitter et al., 2012b), to change the vascular permeability of the air-blood barrier (e.g., enhancement of systemic absorption of therapeutic peptides and proteins) (Amidi et al., 2008; Giantsos-Adams et al., 2011) and to increase the stability of pulmonary surfactant to inhibition (Zuo et al., 2008). Despite the potential of these

Abbreviations: BALF, bronchoalveolar lavage fluid; BSA, bovine serum albumin; d_d, median geometric droplet diameter (based on the volume distribution); DEAPA-PVA, 3-diethylamino-1-propylamine-modified poly(vinyl alcohol); EC_{50}, half maximal effective polymer concentration inducing a halfway drop of the determined d_d from baseline to a minimal size; LDH, lactate dehydrogenase; *p*, probability value; SD, standard deviation.

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promising polymer additives, biocompatibility testing with the physiological environment is currently matter of intense research (Beyerle et al., 2011; Merkel et al., 2011; Pilcer and Amighi, 2010).

The current work investigated the impact of amine-modified poly(vinyl alcohol)s (PVAs) on the process of vibrating-membrane nebulization with special emphasis on the generated aerosol droplet size and output rate characteristics. Preliminary biocompatibility tests of the macromolecular excipients were performed in the pulmonary cell line A549 (i.e., release of lactate dehydrogenase (LDH)). The acute toxicity of lung-delivered amine-modified PVAs was further evaluated in an isolated rabbit lung model by monitoring the organ weight gain kinetics and protein content in the bronchoalveolar lavage fluid (BALF).

2. Materials and methods

2.1. Materials

Mowiol® 3–85 was obtained from Kuraray (Hattersheim, Germany). Amine (i.e., 3-diethylamino-1-propylamine (DEAPA))-modified PVAs were synthesized and characterized as described previously (Nguyen et al., 2010; Unger et al., 2007; Wittmar et al., 2005). These polymers are hereafter abbreviated as DEAPA(z)-PVA, with z displaying the percentage of amine-modified vinyl alcohol units in the polymer chain (degree of amine substitution). Their general chemical structure is illustrated in Figure S1 (Supplementary material). For this study, the following (amine-modified) PVAs were used: Mowiol® 3–85, DEAPA(23)-PVA, DEAPA(32)-PVA and DEAPA(43)-PVA. The molecular weight of employed polymers is listed in Table S1 (Supplementary material). Double-distilled water was acquired from B. Braun (Melsungen, Germany). All other chemicals and solvents used in this study were of analytical grade and used without further purification.

2.2. Preparation of polymer solutions

Polymers were dissolved in double-distilled water for ~12 h, followed by a filtration step (5.0 μm, Cameo 30 N syringe filters, GE Water & Process Technologies, Ratingen, Germany). The current work specifies the polymer concentrations in mg/ml (and μg/ml). Information/results on the polymer concentration in μM can be found in the Supplementary material.

2.3. Physicochemical characterization of polymer solutions

Samples were characterized for density (oscillating density meter, DMA 4100 M, Anton Paar, Graz, Austria), viscosity (Ubbelohde (Type I ($k = 0.01008\ mm^2/s^2$)), Schott, Mainz, Germany), surface tension (Wilhelmy plate, K11-Mk3, Krüss, Hamburg, Germany) and conductivity (FiveEasy®, Mettler-Toledo, Giessen, Germany) at 25.0 ± 0.1 °C.

2.4. Nebulization experiments

Nebulization experiments were carried out using two vibrating-membrane nebulizers (i.e., Aeroneb® Pro (Aerogen, Dangan, Galway, Ireland) and eFlow®rapid (PARI, Starnberg, Germany)) under the following ambient conditions: temperature: 25 ± 1 °C; relative humidity: 60 ± 10%.

2.4.1. Aerosol droplet size

The median geometric diameter (d_d, based on the volume distribution) of the nebulized aerosol droplets was determined by laser diffraction (HELOS, Sympatec, Clausthal-Zellerfeld, Germany) (Beck-Broichsitter et al., 2014a; Beck-Broichsitter et al., 2014e; Beck-Broichsitter et al., 2015; Mitchell et al., 2006). The geometric standard deviation of aerosol clouds was determined from the laser diffraction values and amounted to <2.0 for all investigated formulations. Dose-effect curve characteristics of the polymers on the generated d_d (i.e., half maximal effective polymer concentration (EC_{50} value), which induced a halfway drop of the determined d_d from baseline (d_d ~ 8 μm) to a minimal size (d_d ~ 4 μm)) were calculated using a sigmoidal dose–response function (Origin 7.0, OriginLab, Northampton, USA).

2.4.2. Aerosol output rate

The total aerosol output was determined gravimetrically (BP 211 D, Sartorius, Göttingen, Germany) by weighing the nebulizer unit before and after each nebulization experiment. The weight difference was used to calculate the aerosol output rate in g/min.

2.5. In vitro cell culture

The alveolar epithelial cell line, A549 (ATCC, Wesel, Germany), was selected as a cell culture model for assessment of toxicity (Grabowski et al., 2013). Cells, passages 8–15, were maintained with Dulbecco's modified Eagle Medium (Sigma-Aldrich, Steinheim, Germany) supplemented with 10% of fetal calf serum (Lonza, Köln, Germany) and 2 mM L-glutamine (Sigma-Aldrich, Steinheim, Germany) in an atmosphere of 8.5% CO_2 at 37 °C and a relative humidity of 95%. A549 were seeded at a density of 1.0×10^4 cells/cm^2 in 96-well plates (PSS, Geroldswil, Switzerland) and grown to ~80% confluence (~72 h post-seeding). Cells were then incubated with fresh cell culture medium containing the dissolved polymers (0.01–1.0 mg/ml) for a predetermined period of time (1–24 h) at 37 °C. The amount of LDH released into the cell culture supernatant as a result of cellular damage was quantified using a colorimetric assay for cytotoxicity (Roche, Mannheim, Germany) according to the manufacturer's protocol. The produced red dye was quantified by UV/Vis spectroscopy (Titertek, ICN, Eschwege, Germany) at a wavelength of 492 nm. Results are expressed as relative cytotoxicity compared to the LDH release observed after Triton® X-100 (Sigma-Aldrich, Steinheim, Germany) (1.0 mg/ml in cell culture medium) exposure, which was set to 100%.

2.6. Ex vivo lung experimentation

The acute toxicity of lung-delivered amine-modified PVAs was assessed in an isolated, perfused, and ventilated rabbit lung, which has been previously described in detail (Beck-Broichsitter et al., 2011; Seeger et al., 1994). All experiments were performed in accordance with the German Law on the Use and Protection of Laboratory Animals (*TierSchG*). The Federal Authorities for Animal Research of the "Regierungspräsidium Giessen" (Giessen, Germany) approved the study protocol (17/02/2012, GI 20/10 Nr. A 1/2012).

Briefly, following isolation, rabbit lungs were perfused with 300 ml of Krebs–Henseleit buffer (Serag-Wiessner, Germany) supplemented with 4.0% of bovine serum albumin (BSA) (Carl Roth, Germany) kept at 37 ± 1 °C and ventilated with a gas mixture of 21% O_2, 5.3% CO_2, and 73.7% N_2 (tidal volume: 30 ml, frequency: 30 strokes/min). The isolated lung was placed in a temperature-equilibrated housing chamber (37 ± 1 °C), freely suspended from a force transducer for continuous monitoring of the organ weight (Wägezelle Typ U1, Hottinger Baldwin Messtechnik, Darmstadt, Germany) (Beck-Broichsitter et al., 2014a; Dalla-Bona et al., 2015; Uhlig and Heiny, 1995).

Polymer solution (1.0 ml) was delivered to the airspace of the isolated lung using a MicroSprayer® (Model IA-1B, Penn-Century, Wyndmoor, USA) as previously described (Beck-Broichsitter et al., 2010). This administration procedure allowed for efficient formulation deposition (~95%) within the airspace of the lung model.

At the end of the experiments, a catheter was placed into the trachea and lungs were lavaged with ice-cold isotonic saline (twice with 50 ml each). Recovered BALF was pooled and immediately centrifuged (300g, 15 min). The protein concentration in the supernatant was determined