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Impact of triblock copolymers on the biophysical function of naturally-derived lung surfactant

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ABSTRACT

The current study aimed at investigating the general applicability of triblock copolymers consisting of poly(ethylene glycol) and poly(propylene glycol) (Pluronic®) as excipients for lung delivery. After thorough physicochemical characterization of the diverse polymers, their cytotoxicity was evaluated using alveolar epithelial cells. Next, a naturally-derived lung surfactant was challenged with the distinct triblock copolymers with respect to changes in microstructure, adsorption to the air/liquid interface and dynamic surface tension behavior under bubble pulsation.

Biocompatibility assessment of triblock copolymers in A549 cells demonstrated some cytotoxicity, dependent on the hydrophobicity and dose of the substance applied (effective at ≥0.1 mg/ml). Supplementing triblock copolymers onto Alveofact® had an obvious influence on the aggregation state and surface activity (>25 and >5 mN/m during adsorption and bubble pulsation, respectively) of the lung surfactant. Interestingly, Pluronic® F127, a rather hydrophilic triblock copolymer, showed the most intense effect on the microstructure and biophysical performance of Alveofact®. This is likely due to the synergistic interplay of its low critical micelle concentration and rather high molecular weight, leading to the penetration of lung surfactant film/vesicles and accompanied by a partial replacement of relevant surfactant components from the air/liquid interface.

Overall, suitable compositions and concentrations of triblock copolymers were identified with respect to compatibility with the physiological environment of the deep lungs.

1. Introduction

Triblock copolymers including poly(ethylene glycol)-*block*-poly(propylene glycol)-*block*-poly(ethylene glycol) (PEG-*b*-PPG-*b*-PEG) represent a class of well-established excipients in the field of chemistry, pharmacy and medicine [1–3]. Said polymers form micelles in water, which are capable of solubilizing numerous poorly soluble drug substances [4]. Another interesting feature is the thermo-responsive behavior of many triblock copolymers leading to an *in situ* sol-gel transition upon an increase in temperature, which is currently investigated for diverse controlled drug delivery applications [5,6]. Moreover, PEG-*b*-PPG-*b*-PEGs were frequently employed to coat nanoscale drug delivery systems (PEGylation) with the aim of elevating the colloidal stability [7] and minimizing interactions with the biological environment present at the target site [8,9].

Meanwhile, polymer nanoparticles have been praised for their beneficial performance with respect to prolonged and targeted drug delivery to the lungs [10]. However, numerous nanomaterials have shown a remarkable interplay with the biophysical function of the lining layer found in the deep lung (*i.e.*, lung surfactant) [11–18], due to binding/immobilization of essential surfactant molecules on the surface of nanomaterials [12,19–23]. Recently, it was discovered that coatings of PEG-*b*-PPG-*b*-PEGs protect the surface of polymer nanoparticles from unwanted adsorption of surfactant-associated proteins, leading to significantly reduced impairment/perturbation of lung surfactant function [12]. However, it is important to ensure that triblock copolymers are not present in free form in the formulation, due to an insufficient purification process or a displacement from the surface of the colloidal drug delivery vehicle during application. The amphiphilic nature of many triblock copolymers could condition an accumulation in the air/liquid interface [24] accompanied by potential penetration

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in the lung complex surfactant film (monolayer plus a collection of associated layers) and/or a partial replacement of relevant surfactant components.

Accordingly, this study addressed the question whether the surface activity of lung surfactant is adversely affected by triblock copolymers composed of PEG and PPG as main building blocks. These polymers are commonly utilized for the preparation of diverse drug delivery systems. Prior to incubating a naturally-derived lung surfactant from bovine source (*i.e.*, Alveofact®) with said polymers, their physicochemical properties (*i.e.*, molecular weight, composition and surface activity) and potential cytotoxic activity (*i.e.*, mitochondrial activity in alveolar epithelial cells) were investigated in detail. The microstructure and biophysical activity of Alveofact® in the presence of control and relevant triblock copolymers was then analyzed by optical microscopy and the oscillating bubble technique, respectively. Accordingly, the current observations could be used to provide better understanding for the choice of safe triblock copolymers. Triblock copolymers can be used for masking the surface of nanomedicines in order to reduce the risk of unwanted interactions with the biophysical function of lung surfactant.

2. Materials and methods

2.1. Materials

The control polymers PEG and PPG with a nominal molecular weight of 2 kDa (PEG2k and PPG2k) as well as four ABA (*i.e.*, Pluronic® L61, L121, F68 and F127) and one BAB (*i.e.*, Pluronic® 17R4) triblock copolymer(s) were obtained from Sigma-Aldrich (Steinheim, Germany). Here, PEG and PPG represent the *A* and *B* block, respectively. Alveofact® was procured from Lyomark (Oberhaching, Germany). All other chemicals and solvents used were of analytical grade and used as received.

2.2. Gel permeation chromatography (GPC)

Samples were dissolved in chloroform (~5 mg/ml). After filtration (0.2 μm, Acrodisc®, Pall, Dreieich, Germany), 100 μl of the sample solution was injected into the system, consisting of two columns (PL-gel MIXED-D; 300 × 7.5 mm; bead diameter: 5 μm) from Varian (Amherst, USA) and a differential refractive index detector (SpectraSystem RI-150, Thermo Electron Corp., Waltham, USA). The elution was performed at 30 °C with chloroform at a flow rate of 1 ml/min (toluene as flow-rate marker). PEG standards (Sigma-Aldrich) of known molar masses were employed for calibration.

2.3. Proton nuclear magnetic resonance (^{1}H NMR) spectroscopy

^{1}H NMR spectroscopy was performed in $CDCl_3$ at 25 °C and a polymer concentration of ~10 mg/ml. ^{1}H NMR spectra were recorded on a Bruker Avance 300 spectrometer (Billerica, USA) at 300 MHz. The chemical shift scale was corrected on the basis of the tetramethylsilane signal. The mole% content of ethylene glycol (EG) was determined by a comparison of the integration from the methyl peak (generated from the PPG segment of the molecule) to the total EG/propylene glycol (PG) signal.

2.4. Cell culture

A549 human alveolar basal epithelial cells were obtained from American Type Culture Collection (Manassas, USA). Cells were cultured in Dulbecco's modified eagle growth medium supplemented with 2 mM L-glutamine, 50 U/ml penicillin, 50 U/ml streptomycin and 10% fetal calf serum (all from Sigma-Aldrich) in a humidified atmosphere of 5% CO_2/95% air at 37 °C and sub-cultured twice per week. A549 cells were used from passages 6 to 14 after thawing.

2.5. In vitro *cytotoxicity of control and triblock (co)polymers*

The viability of A549 cells in the presence of the polymers was determined using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay according to Mosmann [25] and Fischer et al. [26]. Briefly, cells were seeded at a density of 1×10^4 cells/well in 96-well plates (TPP, Trasadingen, Switzerland) and grown to sub-confluence (70–80%, approximately 48 h post seeding). Next, the culture medium was discarded and the cells were incubated with serial dilutions of polymers in culture medium for 24 h. Thereafter, the supernatant was replaced by fresh medium containing 0.5 mg/ml MTT. The unreacted dye was removed after 2 h and 200 μl of dimethyl sulfoxide were added to dissolve the formed formazan crystals. Absorption was quantified by photometric analysis (LabSystems Multiskan MS plate reader, ThermoFisher Scientific, Schwerte, Germany) at a wavelength of 570 nm. Results are expressed as relative cytotoxicity (mitochondrial activity) compared to the values observed after exposure to fresh cell culture medium and Triton® X-100 (1 mg/ml in cell culture medium), which were set to 100 and 0%, respectively.

2.6. Preparation of surfactant material

Alveofact® was supplied as a lyophilized powder and resuspended in NaCl solution supplemented with Ca^{2+} by brief sonication (SONOREX DIGITEC, BANDELIN, Berlin, Germany) at a final phospholipid concentration of 50 mg/ml. The exact phospholipid content was determined by a colorimetric phosphorus assay [27] after lipid extraction according to Bligh and Dyer [28].

2.7. Incubation of lung surfactant with control and triblock (co)polymers

Alveofact® stock preparations and polymer stock solutions were combined to meet the desired final phospholipid (*i.e.*, 2 mg/ml) and polymer concentration (*i.e.*, 0.001, 0.01, 0.1 and 1 mg/ml) in isotonic saline containing 2 mM Ca^{2+}. Samples were mixed by vortexing and brief sonication, followed by 30 min of incubation (without shaking) at 37 °C.

2.8. Optical microscopy

The morphology of Alveofact® and Alveofact® incubated with control and triblock (co)polymers (incubation conditions as outlined above) was observed by light microscopy (Diaplan, Leitz, Wetzlar, Germany).

2.9. Biophysical studies

Surface activity of samples was assessed using the oscillating bubble technique (pulsating bubble surfactometer, Electronetics Corp., Amherst, USA) [11,12,21,29]. This technique provided read-outs of the surface tension after film adsorption (γ_{ads}, static measurement) and at a minimum bubble radius during film oscillation (γ_{min}, dynamic measurement). Briefly, after incubation (conditions as outlined above), samples (compositions as outlined above) of 35 μl were transferred to the disposable sample chamber, and the adsorption rate was measured. Therefore, a bubble of minimal radius (0.4 mm) was created and, while maintaining the bubble at that minimal size without pulsation, the pressure difference across the air/liquid interface was monitored. Next, pulsation was started by sinusoidally oscillating the bubble radius between 0.4 and 0.55 mm at 37 °C. The cycling rate was set to 20 cycles/min.