Online Supplement

Nebulised Lipid-Polymer Hybrid Nanoparticles for the Delivery of a Therapeutic Anti-inflammatory microRNA to Bronchial Epithelial Cells

Abbreviated title: miRNA-nanoparticle delivery to bronchial epithelial cells

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MATERIAL AND METHODS

Materials

PLGA with a lactide:glycolide molar ratio of 75:20 (PLGA-7520) was obtained from Wako Chemicals (Neuss, Germany). DOTAP chloride dissolved at 10 mg/ml in chloroform was from Avanti Polar Lipids (Alabaster, AL, USA). Human miR-17-5p and non-targeting NC#1 miRNA mimics (double-stranded RNA oligonucleotides with an inactive antisense strand) and unmodified double-stranded DNA (dsDNA) oligonucleotides were from Integrated DNA Technologies (Coralville, IA, USA). The sequence (sense) of the miR-17 mimic was 5'-CAAAGUGCUUACAGUGCAGGUAG-3' and of its non-targeting equivalent, NC#1, 5'-CAUAUUGCGCGUAUAGUCGC-3'. The sequence of the dsDNA oligonucleotide was 5'-GCGACTATACGCGCAATATGGT-3'. Poly(vinyl alcohol) (PVA) MW: 9000-10000 with an 80% degree of hydrolysis, chloroform, dimethyl ether, trehalose, ethylenediaminetetraacetic acid (EDTA), 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS) and dithiothreitol (DTT) were from Sigma Aldrich (St. Louis, MO, USA). DEVD-amido-4-methylcoumarin (DEVD-AMC) was from Enzo Life Sciences (Farmingdale, NY, USA)

Preparation of miRNA mimic-loaded DOTAP-modified PLGA LPNs

The double emulsion solvent evaporation (DESE) method was used for encapsulation of the miR-17 mimics, NC#1 and the dsDNA oligonucleotides in DOTAP-modified PLGA LPNs as described previously (7, 11). A DOTAP nitrogen-to-oligonucleotide phosphate (N:P) ratio of four was used for encapsulation of the oligonucleotide constructs. Briefly, 12.75 mg PLGA was dissolved in 225 µl chloroform containing DOTAP at a concentration of 10 mg/ml after which the chloroform solvent was purged with nitrogen. The

PLGA:DOTAP solid was dissolved in 250 µl dimethyl ether and 125 µl RNAse-free water containing 100 µM miR-17 mimic, NC#1 or dsDNA oligonucleotide. This mixture was subsequently emulsified with a Branson Sonifier SLPt for a total of 90 s in 1 s pulses at 55% amplitude while submerged in ice water. By means of iodide dosimetry, the sonication output was calibrated to the output used previously (7, 11). After sonication, 1 ml of 2% (w/v) PVA in RNAse-free water was added, and the suspension was vortexed for 1 minute after which it was further sonicated while being chilled by ice water for 60 s in 1 s pulses at 55% amplitude. The dimethyl ether was evaporated off by stirring the suspension in 5 ml 2% (w/v) PVA for 3 hours at room temperature. The LPNs were collected by centrifugation at 26,000·g for 30 minutes at 4°C. To wash the LPNs, centrifugation was repeated three times after sample resuspension in RNase-free water. The LPNs (1 mg) were freeze-dried for 24 hours in 1 ml RNAse-free water in the presence or absence of 4.5% (w/v) trehalose as cryoprotectant.

Physicochemical characterization

The intensity-weighted mean hydrodynamic diameter (*z*-average) and polydispersity index (PDI) were measured by dynamic light scattering using the photon correlation spectroscopy technique. The ζ-potential was determined using laser-Doppler micro-electrophoresis. For both types of measurements, a Zetasizer Nano ZS (Malvern Instruments, Malvern, UK) was employed and the LPNs were diluted at approximately 50 µg/ml in deionised water before the measurements. Prior to measurement, lyophilised LPNs were reconstituted in sterile RNAse-free water with or without 0.9% (w/v) NaCl for 10 minutes at room temperature followed by 3 minutes of gentle vortexing. Calculations were performed using a viscosity and refractive index of 0.8872 cPa and 1.33, respectively.

The LPN yield was measured by weighing a 1:10 lyophilised sample in a pre-weighed, non-static microtube using an MX5 microbalance (Mettler Toledo, Columbus, OH, USA). All materials were electrostatically degaussed prior to weighing to minimize measurement bias and variability caused by electrostatic forces between the microbalance and its load. For determination of encapsulation efficiency (EE) and loading capacity (LC), a 1:10 aliquot (approx. 1 mg) of lyophilised LPNs was dissolved in 200 µl chloroform for 10 minutes and subsequently vortexed at room temperature for 90 minutes after the addition of 500 µl dissociation buffer [10 mM Tris, 1 mM EDTA (pH 7.4), 2 mg/ml heparin and 0.292 mg/ml octyl ß-D-glucopyranoside]. The vortexed emulsion was centrifuged at 26,000·g for 15 minutes at 4°C. The aqueous layer was transferred to a new microtube, and the nucleic acid content was measured using the

Quan-IT microRNA Assay kit (Thermo Fisher Scientific, Waltham, MA, USA) for miRNA mimics or the Qubit dsDNA HS assay kit (Thermo Fisher Scientific) for dsDNA oligonucleotides. The measurements were controlled by performing parallel measurements with stock nucleic acid concentration dissolved in dissociation buffer. The EE was calculated according to:

$$\%EE = \frac{\text{retrieved nucleic acid}}{\text{loaded nucleic acid}} \times 100$$

The LC was calculated according to:

$$LC\ (\mu g_{\text{nucleic acid}}/mg_{\text{LPNs}}) = \frac{\text{retrieved nucleic acid } (\mu g)}{\text{LPNs (mg)}}$$

Aerosol characterization

The mass median aerodynamic diameter (MMAD) and geometric standard deviation (GSD) of the nebulised LPN dispersions were measured using a Next-Generation Impactor (NGI; Copley Scientific, Nottingham, UK) at a flow rate of 15 ± 0.5 l/min. The nebulised LPNs were collected from the NGI stages by elution with 10 ml deionised water, which was subsequently transferred to tubes and kept on ice until counting. Counting of the nanoparticle fractions was performed with the Nanosight NS300 (Malvern) fitted with the flow-cell top plate and a 405 nm laser unit and controlled by the nanoparticle tracking analysis (NTA) software (Malvern). Fractions were diluted with deionised water where necessary to avoid overloading the NTA which otherwise could result in erroneous counting.

The volume median diameter (VMD) of the nebulised LPN dispersions and 0.9% saline was measured using the Spraytec laser diffraction system (Malvern). The VMD is the midpoint droplet size (µm) where half of the volume of spray (aerosol) is in droplets smaller, and half of the volume is in droplets larger than the mean. A 0.25 ml dose volume of both nebulised 0.9% saline and LPN dispersion was used to determine the VMD using real time analysis.

MMAD and VMD measurements were performed in triplicate using three independent samples.

Nanoparticle transfection

Unnebulised LPNs were reconstituted in BEGM for 10 minutes at room temperature, after which the LPN dispersions were gently vortexed for 3 minutes. The LPNs were diluted with BEGM to specified concentrations. The LPNs nebulised in 0.9% saline were specifically diluted with saline and BEGM at ratios normalized across all LPN concentrations. The LPN dispersions were subsequently dosed to NuLi-1 cells grown in 48-well or 96-well plates, either directly or after nebulisation and collection. For knock-down experiments, the cells were incubated with nanoparticle suspensions for 6 hours after which these were replaced with BEGM, and the cells were incubated for an additional 18 hours.

The miRNA mimics were also transfected with the Lipofectamine RNAiMAX transfection agent (Thermo Fisher Scientific) at concentrations of 180 nM and 1.5% (v/v) respectively, as previously optimized (data not shown).

Cell viability assay

NuLi-1 cultures were seeded at a density of 35,000 cells/cm^2 in 96-well plates in BEGM and incubated for 24 hours at 37°C and 5% CO_2. Varying concentrations of reconstituted LPNs in 100 µl BEGM were administered to the cultures at approximately 50% confluency and incubated for 24, 48 and 72 hours. The cell viability was determined using the CellTiter 96 AQueous One Solution Cell Proliferation Assay (Promega, Madison, WI, USA) as per manufacturer's protocol. The A_{490} was measured immediately after media replacement to compensate for any absorbance by the LPNs. The samples were incubated for 60 minutes and the A_{490} was measured again. Cell viability at different nanoparticle concentrations were calculated according to:

$$\text{Cell viability } (\%) = \frac{A_{490\ (t=60,[LPN]_i)} - A_{490\ (t=0,[LPN]_i)}}{A_{490\ (t=60,[LPN]_0)} - A_{490\ (t=0,[LPN]_0)}}$$

with $i =$ nanoparticle concentration (µg/ml) and $t =$ incubation time (minutes)