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Research paper

Rhamnolipids form drug-loaded nanoparticles for dermal drug delivery

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ABSTRACT

Bacterial biosurfactants are nature's strategy to solubilize and ingest hydrophobic molecules and nutrients using a fully biodegradable transport system. Eight structurally defined rhamnolipids were selected and investigated as potential drug carrier systems. Depending on the molecular structures defining their packing parameters, the rhamnolipids were found to form spherical nanoparticles with precisely defined average sizes between 5 and 100 nm, low polydispersity, and stability over a broad concentration range as revealed from dynamic light scattering and electron microscopy. As rhamnolipids were tolerated well by the human skin, rhamnolipid nanoparticles were considered for dermal drug delivery and thus loaded with hydrophobic drug molecules. Using the drug model, Nile red, dexamethasone, and tacrolimus nanoparticles charged with up to 30% drug loading (w/w) were obtained. Nanoparticles loaded with Nile red were investigated for dermal drug delivery in a Franz cell using human skin. Fluoresence microscopy of skin slices indicated the efficient penetration of the model drug into human skin, both into the stratum corneum and although to a lesser extent into the lower epidermis. Rhamnolipid nanocarriers were found to be non-toxic to primary human fibroblasts in a proliferation assay and thus are considered candidates for the dermal delivery of drugs.

1. Introduction

Rhamnolipids are bio-degradable biosurfactants secreted by bacteria like *Pseudomonas aeruginosa* and enable the producing microorganism to solubilize and take up hydrophobic molecules like hydrocarbons utilizing them efficiently as a carbon source [1–4]. As a result, rhamnolipids are used for the bio-remedy of environmental pollution with hydrocarbons, e.g. in the case of crude oil spills [5,6]. Numerous biological activities of rhamnolipids toward bacteria, eukaryotic cells and organisms have been reported [7–9]. Rhamnolipids possess antimicrobial effects [10,11] as well as anti-proliferative activity toward cancer cells [12]. They activate the innate immune system of vertebrates resulting in the secretion of pro-inflammatory cytokines [13,14]. They have been found to exert cell-type specific effects on the proliferation of skin cells [15] and have been demonstrated to induce the production of antimicrobial peptides in skin cells like psoriasin e.g. by the delivery of bacterial proteins to skin cells [16,17]. Due to the observed biological activities of rhamnolipids toward skin cells the compounds have been claimed for clinical studies as a remedy of psoriasis [18]. Accelerated healing with rhamnolipids was observed for burn wounds in mice [19]. Several studies have related the biological activities of rhamnolipids to the molecular structure and to the biophysical properties of these biosurfactants. Various spectroscopic techniques have been applied to this task and some relationships, e.g. between the critical micelle concentration of the compounds and their bioactivity have been established [20].

Considering the numerous biological roles of rhamnolipids, we extended our own investigations on the synthesis [21,22], biophysics [23], and biological activities [13] of these bio-surfactants and started to study their structures in aqueous solutions. Initial experiments revealed that specific rhamnolipids form well-defined and surprisingly stable nanoparticular structures in solution. The initial findings prompted us to investigate the potential of rhamnolipids as nanocarriers for dermal drug delivery. For the purpose nanoparticles of various rhamnolipids were generated, analyzed and loaded with the hydrophobic drug molecules dexamethasone or tacrolimus, or the drug model Nile red. Finally, delivery of the model drug was investigated in a skin model.

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2. Materials and methods

Rhamnolipids **RL-3 — RL-8** were prepared by chemical synthesis as described earlier [21] or purchased (**RL-1,-2)** from Sigma-Aldrich (St. Louis, Missouri, USA). Nile red, dexamethasone, and tacrolimus were from Sigma-Aldrich Co. LLC, St. Louis, Missouri, USA. Solvents (acetonitrile, DMSO) were acquired in HPLC grade as well from Sigma-Aldrich and VWR and were used as received.

2.1. Nanoparticle generation protocol A

Rhamnolipids (8 mg) were dissolved in DMSO (4 µl). Hydrophobic drug (10 mg) was suspended in the rhamnolipid-DMSO solution (0.5 µl), vortexed, and heated in an ultrasound bath at 35 °C for 15 min. The resulting suspension was stored at room temperature overnight before 100 µl of PBS-buffer (pH 7.4) were added. The final stock solution was obtained after another cycle of vortexing, heating during ultra-sonication and filtration through a recovered cellulose syringe filter. Dilution series were prepared with PBS buffer, which was filtered through a 0.2 µm PTFE syringe filter, obtaining solutions with final concentrations between 5 µg/l and 10 g/l.

2.2. Nanoparticle generation protocol B

Rhamnolipids (10 mg) were dissolved in PBS-buffer (7.4 pH, 1 ml). In 100 µl of this solution 10 mg of the hydrophobic drug (Nile red, dexamethasone, or tacrolimus) were suspended. The resulting solution was vortexed, heated in an ultrasound bath at 35 °C for 15 min and was stored at room temperature overnight. The final stock solution was obtained after another cycle of vortexing, heating during ultra-sonication and filtration through a recovered cellulose syringe filter. Dilution series were prepared with PBS buffer, which was filtered through a 0.2 µm PTFE syringe filter, obtaining solutions with final concentrations between 5 µg/l and 10 g/l.

2.3. Particle size analysis by dynamic light scattering (DLS)

The size of rhamnolipid nanoparticles were analyzed by dynamic light scattering experiments (DLS) on a Zetasizer Nano from Malvern using a 4 mW He-Ne laser (633 nm and 532 nm) with a fixed detector angle of 173°. All measurements were performed at 25 °C and within disposable dust-free light scattering micro cuvettes (40 µl). Each DLS experiment consisted of 16 separate runs. Measured data were averaged and evaluated using Zetasizer Software from Malvern Instruments Ltd., Malvern, United Kingdom. PDI values were calculated as the ratio of the square of the standard deviation and the square of mean particle diameters [24].

2.4. Transmission electron microscopy and scanning electron microscopy

Rhamnolipid PBS buffer solutions of 2 mg/ml and 1 mg/ml were dropped on a grid, the solvent was evaporated at room temperature and subsequently analyzed with a Field Emission Scanning Electron Microscope (FE-SEM) of Hitachi High-Technologies Europe GmbH, Krefeld, Germany (UHR Cold-Emission FE-SEM SU8030).

2.5. Determination of the drug loading of nanoparticles

Calibration curves were established via LCMS measurements of dilution series of hydrophobic drugs in acetonitrile. Each of eight dilutions ranging from 1 mg/l to 5 g/l was measured tree times followed by quantification via integration of the peaks in the extracted ion chromatogram (EIC) using HPLC/MSD ChemStation (Agilent Technologies Inc., Santa Clara, Calfornia, USA).

HPLC/MS measurements were conducted with an HPLC of the Agilent 1100 series (G1956B) equipped with a diode array detector and coupled with a single quadrupole mass spectrometer with an ESI source from Agilent technologies.

The measurements of rhamnolipids loaded with dexamethasone or Nile red were conducted using a Pursuit XRs-C8 (3.0 µm, 4.6 × 100 mm) column from Agilent Technologies. The eluents water and acetonitrile (ACN) with 0.1% formic acid were used in a linear gradient (10–99% ACN in 4.5 min). Measurements of rhamnolipids loaded with tacrolimus were conducted with a LUNA C18 (2) (3.0 µm, 4.6 × 100 mm) column from Phenomenex Ltd. (Aschaffenburg, Germany) due to an improved signal-to-noise-ratio. The eluents water and acetonitrile (ACN) with 0.1% formic acid were used in a linear gradient (60–99% ACN in 5.5 min). Loadings were determined as the average of tree measurements.

2.6. Skin penetration experiments

Characterization of dermal drug delivery was evaluated by using rhamnolipid nanoparticles loaded with the fluorescence dye Nile red using a Franz cell set-up as described earlier [25]. Prior to the experiments, human skin was thawed. Discs of 2 cm diameter were punched and mounted onto Franz cells (static-type, volume 12 mL, diameter 15 mm (PermeGear Inc., Bethlehem, PA, USA). The horny layer was facing the air and the dermis had contact with the receptor fluid PBS pH 7.4 of 33.5 °C. The skin surface temperature was 32 °C. It was stirred at 500 rpm. After 30 min, 35 µL of Nile red loaded rhamnolipids with a concentration of 2 or 5 mg/ml were applied onto the skin surface and remained there for 6 h. Thereafter, the skin was removed and the surface was gently rinsed with PBS. Subsequently skin regions were punched and embedded in tissue freezing medium (Jung, Nussloch, Germany). They were stored in Peel-Aways (Polysciences Europe GmbH, Eppelheim, Germany) at −80 °C and cut in vertical slices of 8 µm thickness by a freeze microtome Frigocut 2800 N (Leica Mikrosysteme Vertrieb GmbH, Bensheim, Germany). Nile red residues penetrating into the dermal layers of these slices were determined using the fluorescence microscope BZ-8000 (Keyence Deutschland GmbH, Neu-Isenburg, Germany).

2.7. Cell proliferation assay

The cell proliferation assay was carried out with primary human dermal fibroblasts isolated from adult skin. Primary human dermal fibroblast cells were derived from three adult donors, pooled and cultured in DMEM (PAA Laboratories GmbH, Cölbe, Germany) supplemented with 10% FBS for four passages. Cells were seeded at the density of 2 × 105 per well and incubated with a serial concentration with of rhamnolipids RL-1 and RL-2 (0.1 to 0,003125 mg/mL) for 24 h. Alamar Blue® reagent was incubated in medium with 10% v/v to determine the cell viability percentages. Absorbance of samples was measured in triplicates at 570 nm and 600 nm wavelengths using the TECAN Safire II microplate reader (Tecan Systems, Inc., Männedorf, Switzerland) [26–28].

3. Results and discussion

Rhamnolipids **RL-1—RL-8** differing with respect to the chain length, number of sugar residues, the stereochemistry and chemical nature of the head group were obtained from chemical synthe-