Colloids and Surfaces B: Biointerfaces 143 (2016) 301–308

Contents lists available at ScienceDirect

Colloids and Surfaces B: Biointerfaces

journal homepage: www.elsevier.com/locate/colsurfb

Inhalable polymer-glycerosomes as safe and effective carriers for rifampicin delivery to the lungs

Virginia Melis[a], Maria Letizia Manca[b,*], Enrica Bullita[c], Elena Tamburini[c], Ines Castangia[b], Maria Cristina Cardia[b], Donatella Valenti[b], Anna Maria Fadda[b], José E. Peris[a], Maria Manconi[b]

[a] Dept. Farmacia y Tecnologia Farmaceutica, University of Valencia, 46100-Burjassot, Valencia, Spain
[b] Dept. Scienze della Vita e dell'Ambiente, CNBS, University of Cagliari, Cagliari, Italy
[c] Dept. Scienze Biomediche, University of Cagliari, Cagliari, Italy

ARTICLE INFO

Article history:
Received 2 February 2016
Received in revised form 13 March 2016
Accepted 15 March 2016
Available online 16 March 2016

Keywords:
Phospholipid vesicles
Glycerol
Trimethyl chitosan chloride
Sodium hyaluronate
Pulmonary nebulization
In vivo distribution
A549 cells

ABSTRACT

Rifampicin loaded glycerosomes, vesicles composed of phospholipids, glycerol and water, were combined with trimethyl chitosan chloride (TMC) to prepare TMC-glycerosomes or, alternatively, with sodium hyaluronate (HY) to obtain HY-glycerosomes. These new hybrid nanovesicles were tested as carriers for pulmonary delivery of rifampicin. Glycerosomes without polymers were also prepared and characterized. All vesicles were similar: they were spherical, multilamellar and able to incorporate good amount of rifampicin (EE% ~ 55%). The addition of the polymers to the formulations allowed an increase of mean diameter. All the glycerosomes, in particular HY-glycerosomes, were able to deliver the drug to the furthest stages of the Next Generation Impactor and the aptitude of the vesicles to be nebulized was always higher than that of drug dispersion. Rifampicin nanoincorporation in vesicles reduced the *in vitro* drug toxicity on A549 cells, as well as increased its efficacy against *Staphylococcus aureus*. Finally, the *in vivo* biodistribution and accumulation, evaluated after intra-tracheal administration to rats, confirmed the improvement of rifampicin accumulation in lungs.

1. Introduction

Recurring pulmonary infections are often associated to different chronic pathologies, like asthma, cystic fibrosis, and immunocompromised syndromes, making necessary the frequent use of antibiotics as standard therapy. Lung infections are usually treated by systemic administration (orally or parenterally) of high doses of antibiotics leading to several adverse effects, especially when a potent drug is employed. Unfortunately, the recurrent treatment of these chronic infections usually induces bacterial resistance, which is responsible for the treatment failure. When drug resistance occurs during treatment with an antibiotic, the administration of alternative molecules, such as rifampicin, one of the most potent and broad-spectrum antibiotic might often control and eradicate the resistant bacteria. In addition, its pulmonary delivery may represent a valid alternative since it can be delivered directly to the site of infection, circumventing the hepatic first-pass metabolism, maximizing the local concentration and efficacy, and minimizing the systemic distribution and related side effects [1–3]. A promising strategy to improve pulmonary distribution and accumulation of drugs is their loading into suitable micro and nanocarriers, particularly able to improve aerosol performances and local bioavailability of drugs in a single solution [4]. Among others, liposomes composed of naturally occurring phospholipids at an appropriate dose should not pose a toxicological risk to this organ. In addition, their peculiar structural properties allow to load in their aqueous compartment, or in the phospholipid bilayer, or at the bilayer interface drugs with different lipophilicity grades. Nevertheless their versatility, their use is limited by vesicle physical unstability and fast metabolizing, for this reason many technological efforts have been carried out to improve their stability and lung affinity. In particular, an increase of vesicle stability and lung accumulation was obtained by coating them with different natural and synthetic polymers [5–8]. Alternatively, glycerosomes, phospholipid vesicles containing high amount of glycerol designed and tested for skin administration of diclofenac and quercetin, demonstrated optimal drug delivery properties as well as a better stability than conventional liposomes [9–11].

* Corresponding author.
E-mail address: mlmanca@unica.it (M.L. Manca).

http://dx.doi.org/10.1016/j.colsurfb.2016.03.044
0927-7765/

Taking into account the innovation introduced by this nanocarrier, in the present work, glycerosomes, obtained using 50% of glycerol in the water phase, were used to incorporate rifampicin and their formulation was further modified by alternatively adding two different polymers, sodium hyaluronate (HY) or trimethyl chitosan chloride (TMC), to obtain hybrid HY-glycerosomes and TMC-glycerosomes, respectively. These vesicles were successfully used to improve the pulmonary deposition of curcumin and, here, were tested as carrier for rifampicin lung delivery [12]. In both cases these systems were designed to obtain more stable systems, able to efficiently reach and accumulate in the lungs, thus, improving local drug bioavailability and prolonging its residence time. Physico-chemical properties of glycerosomes and polymer-glycerosomes were evaluated and their aptitude to be nebulized was studied using a Pari SX® air jet nebulizer connected to the Next Generation Impactor (NGI). Nebulizers are considered as appropriate systems to assess the nanosystem nebulization capability, because they are able to generate relatively large volumes of aerosol and, additionally, can deliver almost all therapeutic classes of drugs [13]. Since it is well known that pulmonary drug delivery system must be safe and not irritant, biocompatibility of studied formulations was evaluated *in vitro* using pulmonary epithelial cells (A549). Moreover, the *in vitro* activity of each formulation was tested against *Staphylococcus aureus* (*S. aureus*), chosen as representative pathogen that can colonize the human nasal tract and lungs causing infections as well as developing bacterial resistance in both hospital- and community-acquired pneumonia. Finally, the *in vivo* rifampicin accumulation in lungs and biodistribution provided by the polymer-glycerosomes were evaluated using Wistar rats after intratracheal administration.

2. Material and methods

2.1. Material

Phospholipon®90G (P90G), a commercial mixture, containing phosphatidylcholine, phosphatidylethanolamine, fatty acids and triglycerides, was kindly supplied by AVG S.r.l. (Garbagnate Milanese, Italy) and Lipoid GmbH (Ludwigshafen, Germany). Sodium hyaluronate low molecular weight (200–400 kDa) was purchased from DSM Nutritional Products AG Branch Pentapharm (Aesch, Switzerland). Rifampicin (RFP), chitosan (low molecular weight 50–190 KDa and 75–85% deacetylated), sodium iodide, methyl iodide, *N*-methylpyrrolidinone, acetone, glycerol and all the other products were of analytical grade and purchased from Sigma-Aldrich (Milan, Italy). All the cell culture reagents were purchased from Life Technologies Europe (Monza, Italy).

2.2. Preparation and characterization of trimethylchitosan chloride

The trimethyl chitosan chloride was synthesized slightly modifying the procedure reported by Wang et al. [14]. Chitosan, sodium iodide and *N*-methylpyrrolidinone have been vigorously stirred at controlled temperature (60 °C) for 20 min. NaOH solution (11 ml, 15% w/w) and methyl iodide (12 ml) were added and the mixture was maintained for 60 min under vigorous stirring at 60 °C, collected to a refrigerator to avoid the CH_3I evaporation. To control the chitosan degree of quaternization, methyl iodide (5 ml) and NaOH (10 ml, 15%) were added again. The mixture was kept at 60 °C for 6 h and finally at room temperature overnight under stirring. The reaction mixture was concentrated and purified by dialysis. To change the I-counterions with Cl-counterions, the polymer was treated with a NaCl solution (10%) at room temperature overnight and then freeze-dried. ^{1}H NMR spectroscopy was performed on a Varian INOVA-500, (Oxford, USA). The ^{1}H NMR spectra were recorded at 27 °C, using deuterium oxide as solvent. All measurements were done with water suppression. The percentage of quaternization degree (DQ%) of the trimethyl chitosan chloride was calculated using the equation 1 already reported by Verheul et al.: [15]

$$DQ\% = \left(\frac{[N(CH3)3]}{[H1]} x \frac{1}{9}\right) x100 \quad (1)$$

where $[N(CH_3)^3]$ is the integral of the trimethyl amino group and H^1 is the integral of the proton on the C^1 of the glycoside ring.

2.3. Vesicle preparation and purification

Rifampicin (100 mg) and phospholipids (1.8 g) were weighed in a glass flask, hydrated with 10 ml of glycerol/water mixture (1/1 v/v) to obtain glycerosomes. Alternatively, the same amount of drug and lipids were hydrated with 10 ml of sodium hyaluronate or trimethyl chitosan chloride dispersion (0.1%) in glycerol/water (1/1 v/v) to obtain HY-glycerosomes and TMC-glycerosomes, respectively. All the obtained dispersions were sonicated with a high intensity ultrasonic disintegrator (Soniprep 150, MSECrowley, London, United Kingdom) using the same experimental conditions and cycles: 25, 10, 15 and 25 cycles (2 s on and 2 s off, 15 μm of probe amplitude) with pause of 2 min after each cycle group to promote the cooling of the samples [9]. Samples were purified from the non-incorporated drug by dialysis, putting each dispersion (2 ml) into a dialysis tube (Spectra/Por® membranes: 12–14 kDa MW cut-off, 3 nm pore size; Spectrum Laboratories Inc., DG Breda, The Netherlands) and dialyzing against water at room temperature for 2 h (by replacing the water every 30 min). The used water (8 l) was appropriate to allow the dissolution and consequent removal of the unentrapped rifampicin which was <20 mg (solubility in water pH 7.3: 2.5 mg/ml), and to avoid the destabilization of the vesicular suspension (e.g., osmotic swelling and vesicle fusion) as well [6].

2.4. Vesicle characterization

Vesicle morphology of each formulation was checked by a Jem1010, Jeol, transmission electron microscope (TEM) [9]. Size distribution (average diameter and polydispersity index, PI) and zeta potential of the samples were measured using a Zetasizer nano (Malvern Instrument, London, United Kingdom). All the vesicle dispersions were very concentrated and colored (red), then, before the analysis, each sample (100 μl) was diluted using a water/glycerol mixture (1/1 v/v, 10 ml) to avoid the variation of the intervesicle medium. Samples were backscattered by a helium–neon laser (633 nm) at an angle of 173° and a constant temperature of 25 °C. The size and size distribution were estimated as a function of the intensity of the obtained signal. Zeta potential was assessed using the Zetasizer nano-ZS by means of the M3-PALS (Phase Analysis Light Scattering) technique, which measures the particle electrophoretic mobility in a thermostated cell.

Entrapment efficiency (EE%) was expressed as the percentage of the amount of incorporated drug versus that initially used. Drug amount was determined by HPLC, after dilution of the sample with methanol (1/1000). Drug content was quantified at 337 nm using a chromatograph Alliance 2690 (Waters, Milan, Italy) equipped with a photodiode array detector and a computer integrating apparatus (EmpowerTM 3). The column was a Symmetry C18 column (3.5 μM, 4.6 × 100 mm, Waters) and the mobile phase was a mixture of water/acetonitrile (30:70, v/v), delivered at a flow rate of 0.5 ml/min.

Quantitative determination of phospholipids was carried out using the Stewart assay [16]. Vesicle dispersions (10 μl) were added