Inhalable Polymer-Glycerosomes as Safe and Effective Carriers for Rifampicin Delivery to the Lungs

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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.

Keywords: Phospholipid vesicles; glycerol; trimethyl chitosan chloride; sodium hyaluronate; pulmonary nebulization; *in vivo* distribution; A549 cells.

1. Introduction

Recurring pulmonary infections are often associated to different chronic pathologies, like asthma, cystic fibrosis, and immuno-compromised 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]

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 minutes. NaOH solution (11 ml, 15% w/w) and methyl iodide (12 ml) were added and the mixture was maintained for 60 minutes 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 hours 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]

$$DQ\% = \left(\frac{[N(CH3)3]}{[H1]} \; x \; \frac{1}{9}\right) x \; 100 \qquad \text{eq. 1}$$

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 seconds on and 2 seconds 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, 4.6x100 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 to the reagent and obtained solutions were maintained at room temperature for 30 min and then analysed at 485 nm using a UV spectrophotometer (Spectrometer Lamba 25, Perkin Elmer). Aggregation efficiency (AE%), which expresses the actual amount of P90G aggregated in vesicular structures, was calculated as the percentage of the P90G amount initially used.

2.5. Nebulization and aerodynamic behaviour of formulations

A Pari SX® air jet nebulizer attached to a Pari TurboBoy® compressor (Pari GmnH, Starnberg, Germany) was used and connected to the Next Generation Impactor (NGI, Apparatus E, Eur. Ph 7.2, Copley Scientific Ltd., Nottingham, United Kingdom). The flow rate was adjusted to 15 l/min. Samples (3 ml) were placed in the nebulizer and aerosolized for about 15 min, [17] directly into the throat of the NGI. At the end of the experiment, the drug amount deposited in each stage of the impactor and the residual (undelivered) was collected, using methanol, in a glass vials and analyzed by HPLC. Deposition performances were evaluated calculating: 1) the Emitted Dose (ED%), expressed as the percentage of drug recovered in the NGI versus the amount of drug placed in the nebulizer; 2) the Fine Particle Dose (FPD), which represents the amount of drug contained in droplets of size less than 5 μm; and 3) the Fine Particle Fraction (FPF), which represents the percentage of droplets with aerodynamic diameter less than 5 μm. The total mass recovered from each stage was calculated as the sum of all recovered deposits. Mass median aerodynamic diameter (MMAD) ± geometric standard deviation (GSD) values were calculated not including the mass deposited in the induction port because of the unavailability of a precise upper size limit for particles deposited in this section. [18] The cumulative amount of rifampicin-containing droplets with a diameter lower than the stated size of each stage was plotted as a percentage of recovered

drug versus the cut-off diameter. Finally, the MMAD of the particles was extrapolated from the graph according to the Eur. Ph. 7.2.

2.6. *In vitro* cytotoxicity studies (MTT Assay)

Human A549 alveolar cells (at passage 40) were grown in Dulbecco's Modified Eagle Medium supplemented with 10% fetal bovine serum, 1% penicillin/streptomycin and 1% fungizone under 5% CO_2 and 100% humidity at 37°C. Before the experiment, cells were placed into 96-well plates at a density of 7.5×10^3 cells/well and after 24 h were treated for 4, 8, 24 and 48 h with rifampicin in dispersion or loaded in vesicles and properly diluted to have five different concentrations (100, 50, 20, 10, 1 µg/ml). After each incubation time, cells were washed 3 times with PBS and cells viability was determined by MTT [3(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay adding to each well the MTT reagent (200 µl of MTT reagent 0.5 mg/ml in PBS) and, after 2/3 h, dimethyl sulfoxide was added to dissolve the formed formazan crystals. The sample intensity was measured spectrophotometrically at 570 nm with a microplate reader (Synergy 4, ReaderBioTek Instruments, Bernareggio, Italy). [19] All experiments were repeated at least three times and in triplicate. Results were shown as percentage of cell viability in comparison with non-treated cells (100% viability).

2.7. Measurement of antibacterial activity of rifampicin formulations

The antibacterial activity was evaluated by determining the Minimum Inhibitory Concentration (MIC) using the broth microdilution procedure. *S. aureus* (DSM 2569) was grown on Tryptic soy agar (Pronadisa-Conda, Madrid, Spain) at 37°C for 24 h then, suspensions were prepared in cation-supplemented Mueller-Hinton (Sigma-Aldrich, Milan, Italy) broth and inoculated at $5x10^4$ cfu/well. The rifampicin formulations were diluted in water up to 1 g/l. Serial twofold dilutions of each formulation were prepared over the range of 2-0.008 mg/l in MH broth immediately before testing. Each test tube containing a diluted formulation was inoculated with the *S. aureus* suspension and incubated aerobically at 37°C for 24 h. MIC corresponds to the lowest compound concentration that

show the complete growth inhibition compared with the growth control. Ciprofloxacin was used as reference compound and results were within the recommended limits (data not shown).

2.8. *In vivo* drug biodistribution studies

Healthy male Wistar rats, 2-3 months old and weighing 280–310 g, were used in this study. All animals were obtained from the animal facilities of Faculty of Pharmacy, University of Valencia, and were kept in a clean room at a temperature of 23±1ºC, a relative humidity of 60% and a light-dark cycles of 12 hours. Rats were fed a standard laboratory diet obtained from Harlan Laboratories Inc. (Barcelona, Spain) and had *ad libitum* access to water. All studies were performed in accordance with European Union regulations for the handling and use of laboratory animals. Rats were randomized into 3 groups of 6 animals and were intratracheally administered with a dose of 0.85 mg of rifampicin (0.15 ml of drug dispersion, HY-glycerosomes or TMC-glycerosomes). Before to the intratracheal instillation of samples rats were cannulated in the jugular vein to facilitate blood sample collection using a procedure reported previously [20]. After surgery, rats were allowed to recover from the anesthesia and blood samples (200 µl) were taken with heparinized syringes connected to the jugular vein cannula at 0.5, 1, 2, 3, 4, 6, 8, and 24 h after dosing. Blood samples were centrifuged at 1500 *g* for 5 min and the supernatant plasma was stored at −20°C until analysis. Rats were sacrificed after the last blood sampling (24 h) and liver, lungs and trachea were excised and stored at −20°C. The drug concentration in plasma and tissue samples was determined by HPLC after their deproteinization with acetonitrile. Tissue samples were homogenized in a mixture of water and acetonitrile (30:70, v/v) using a Ultra-Turrax® T25 homogenizer (IKA® Werke GmbH & Co. KG, Staufen, Germany) and, after centrifuging the mixture at 12500 *g* for 10 min, the supernatant was injected into the HPLC. The drug concentration in the lungs was separately determined in left inferior lobe, right inferior lobe, right superior lobe and, additionally, the total concentration in the whole lungs was calculated after determining the concentration in the rest of lung tissue. The analysis was carried out at 334 nm using a Waters

Novapak C18 column and a mobile phase composed of acetonitrile and 50 mM NaH_2PO_4 (30:70, v/v) delivered at a flow rate of 1 ml/min.

2.9. Statistical analysis of data

Data analysis was carried out with the software package R, version 2.10.1. Results were expressed as the mean±standard deviation. Multiple comparisons of means (ANOVA) were used to substantiate statistical differences between all groups, while Tukey's and Student's t-test was used for comparison between samples. Significance was tested at the 0.05 level of probability (p).

3. Results

3.1. Synthesis and characterization of trimethyl chitosan chloride

The purified polymer was characterized and its degree of quaternization was determined by ^{1}H-NMR spectroscopy, using the procedure of water suppression (Figure 1). ^{1}H-NMR (500 MHz, D_2O): δ 2.15 (s, CH_3-C=O-NH), 2.87 (s, $N(CH_3)_2$), 3.43 (s, $N(CH_3)_3$); 3.50 (s, 6-OCH_3), 3.59 (s, 3-OCH_3), 3.82-4.58 (m, H_2, H_3, H_5, H_6), 4.82 (br.s , H_1, $N(CH_3)_2$), 5.16-5.85 (m, H_1, GluNAc and $N(CH_3)_3$). The degree of quaternization of the obtained trimethyl chitosan chloride was ~70% and it was soluble in water at the used concentration.

3.2. Preparation and characterization of vesicles

Vesicles were prepared by an environmentally-friendly preparation method which avoided multiple steps, the use of environmental pollutant organic solvents, and additionally energy waste. [21,22] In fact, they were obtained by directly hydrating phospholipids and drug mixture with glycerol/water or glycerol/water/polymer dispersion and finally sonicating them. To achieve the best features of vesicles a pre-formulation study, mainly focused on the assessment of sonication cycles, was carried out. Results underline that to obtain small and homogenous vesicles, at least 75 cycles were needed, but was necessary to intercalate some pauses after 15-25 cycles to facilitate the dispersion cooling. Finally, a common procedure involving 25, 10, 15 and 25 cycles (2 sec ON and 2 sec OFF each) intercalated with pauses of two minutes, was selected for the three formulations. Such method

ensured the formation of small and homogeneous vesicles even in the presence of the polymer. Samples were deeply characterized evaluating morphology, size distribution, zeta potential and structure of vesicles. TEM analysis confirmed the presence of closed lamellar structures in all the dispersions. Glycerosomes were multilamellar vesicles with spherical and regular shape. HY- and TMC-glycerosomes were multilamellar and spherical too, but they appeared slightly aggregated and irregular, Figure 2.

Size and size distribution were evaluated by dynamic laser light scattering measurements. Glycerosomes showed the smallest size (~57 nm) and the most fairly homogeneous system (PI = 0.36), while HY-glycerosomes and TMC-glycerosomes were slightly larger (~78 and ~108 nm, $p<0.05$ versus liposomes) and with a higher polydispersity index (~0.46, $p<0.05$ versus liposomes), probably due to the free polymer still present in the medium, which may be responsible for the heterogeneity of the dispersions. Anyway, results were always repeatable as confirmed by the low standard deviations of mean diameters obtained from at least six repetitions. [23] Zeta potential was highly negative especially for glycerosomes and HY-glycerosmes (~ -40 mV), therefore, indicative of great stability against aggregation and fusion. The zeta potential value decreased in TMC-glycerosomes (~ -30 mV, $p<0.05$ versus others) as a consequence of the partial neutralization of the phosphatidylcholine negative charges by the polycation polymer.

The entrapment efficiency (EE) was around 50% and aggregation efficiency (AE) was approximately 66% for all the formulations ($p>0.05$ among all groups). AE seems not to be affected by the presence of polymers confirming that they do not interfere with phospholipid aggregation.

3.3. Aerodynamic properties of samples

Samples were nebulized using the PariSX® air jet nebulizer connected to the NGI in order to evaluate the drug deposition and the aerodynamic diameter of each formulation. Rifampicin dispersion in glycerol/water was used as control. It is well known that the process of jet nebulization involves repeated cycles of aerosol formation and recapture in the nebulizer reservoir

before the formulation leaves the device. During this process, considerable shearing forces are applied to the tested formulations and for this reason the carrier stability plays a key role for its use as a system for pulmonary administration. [24] Nebulization was performed for 15 min nevertheless, the nebulizer content was not completely aerosolized in all cases being the total mass output (TMO) always lower than 100%. Glycerosomes and polymer-glycerosomes showed the highest TMO values (~94% and ~87%) while the amount of nebulized drug provided by the dispersion was lower (~64%, $p<0.05$ versus others). This result is probably due to the low water solubility of the drug, which aggregated in large particles that were nebulized less efficiently. In order to predict the lung deposition of the tested formulations, the FPD and the FPF were also measured (Table 2). The best rifampicin deposition was provided by HY-glycerosomes (~7.1 mg and ~77%, $p<0.05$ versus others) followed by both glycerosomes and TMC-glycerosomes which allowed ~65% ($p>0.05$ glycerosomes versus TMC-glycerosomes) of rifampicin reaching the furthest stages of the impactor mimicking the deeper part of the respiratory tree. Using the rifampicin dispersion, the amount and the percentage of drug deposited in the last stages of the NGI was significantly lower, ~2 mg and ~44% ($p<0.01$ versus others) respectively. All formulations showed a good aptitude to be nebulized as confirmed also by their aerodynamic diameter, which was ~4 μm ($p>0.05$) for both glycerosomes and polymer-glycerosomes (Table 2). *In vitro* nebulization results confirmed that all prepared formulations, especially HY-glycerosomes, were able to improve rifampicin lung deposition with respect to the drug dispersion.

3.4. *In vitro* cytotoxicity of formulation on A549 cells

MTT assay as cell viability study was performed to evaluate the toxicity of rifampicin loaded vesicles being drug dispersion used as reference (Figure 3). The effect of samples on the viability of A549 cells was evaluated as a function of time and rifampicin concentration. At each tested time, the viability of cells treated with all vesicle formulations was always ≥85% ($p>0.05$ versus times and formulations); the viability was not dose-dependent or time-dependent, only at 4 h the cell

viability was slight higher than that obtained at the other times. Different was the behavior of cells treated with the drug dispersion, which caused a decrease of cell viability as a function of both drug concentration and time. In this case, the dose-depending decrease was more evident at 24 and 48 h when cell mortality reached ~80% ($p<0.01$ versus 4 and 8 h).

3.5. Antibacterial activity of samples

S. aureus was selected as representative, common and causative agent of airway disease, which can be also the most serious multidrug-resistant pathogen able to produce recurring infections. The formulation ability to improve drug efficacy against this pathogen was probed. Rifampicin dispersion displayed a MIC of ~0.016 mg/l, confirming its high ability to kill *S. aureus.* As expected, rifampicin incorporation in all the vesicles, led to an even higher bactericidal activity (doubled) and MIC was significantly reduced (~0.008 mg/l, $p<0.05$ versus dispersion).

3.6 *In vivo* biodistribution studies

Promising results obtained in *in vitro* studies using rifampicin loaded polymer-glycerosomes, led us to carry on further tests to evaluate their behavior *in vivo*. Due to the high similarity of *in vitro* performances provided by rifampicin loaded glycerosomes and TMC-glycerosomes, *in vivo* studies were carried out using the polymer-glycerosomes and comparing them with the free drug dispersion. Samples were delivered by intratracheal administration to rats and the drug retention in the trachea, lungs, liver and plasma was measured.

Rifampicin plasma concentrations obtained after administration of the drug dispersion and TMC-glycerosomes were very similar ($p>0.05$): Cmax value was reached after 30 minutes, then plasma concentration progressively declined up to 8 h ($p<0.05$ versus 30 minutes) and at 24 h the drug amount was below the limit of quantification. The highest values of rifampicin plasma concentration were obtained administering HY-glycerosomes, which provided higher ($p<0.05$ versus others at 2 h) and slightly delayed Cmax. Moreover, rifampicin plasma levels were maintained almost constant till the 4 h and, then, decreased quickly to values that, however, were

always slightly higher than those obtained with the free drug or TMC-glycerosome loaded drug. Anyway, the systemic distribution of the rifampicin was low: the maximum was found after HY-glycerosome administration (0.67±0.47 μg/ml, $p<0.05$ versus others), while ~0.4 μg/ml reached the plasma when rifampicin dispersion and TMC-glycerosomes were used. Concerning the drug retained in the lungs at 24 h after dosing, HY-glycerosomes allowed the highest drug retention ($p<0.05$ versus others), while TMC-glycerosomes led to its minimal accumulation ($p<0.05$ versus others) and, practically, no retention was observed following free drug administration (Figure 4). In particular, after HY-glycerosome administration, rifampicin was mainly found in the inferior lobe of the right lung (~14 μg/g), followed by the superior lobe of the right lung (~11 μg/g) and, finally, the smallest quantity was found in the inferior lobe of the left lung (~8 μg/g). Using TMC-glycerosomes, the values were significantly lower (i.e. ~0.6, 0.7 and 0.8 μg/g, respectively). Only a very small amount of drug was found in the trachea after HY-glycerosome administration, and it was also detected in the liver at 24 h but in very low concentration (≤1.3 μg/g), which was negligible in comparison with that found in lungs when HY-glycerosomes were tested (Figure 5).

4. Discussion

Chronic pulmonary infections are highly frequent and their recurrent treatment with antiinfectious drugs leads to the development of resistant strains. [4] Nowadays, the new research strategies on infectious diseases are aimed at improving the therapeutic index while reducing bacteria resistant to currently available drugs, by using nanotechnology. [25] To this purpose, biocompatible nanovesicular systems loading rifampicin were designed in this work to improve drug bioavailability in lungs. Hybrid polymer-glycerol/phospholipid vesicles were prepared combining the glycerosomes with two bioadhesive and safe polymers by using a one-step and environmentally-friendly method, and biocompatible components: P90G, a commercial mixture of phosphatidylcholine and other lipids, glycerol, water, sodium hyaluronate and trimethyl chitosan chloride. The last one was synthesized starting from chitosan to convert it in a water soluble

polymer. In fact, chitosan is a muchoadhesive and biocompatible polymer widely used for pulmonary drug administration. [25] Unfortunately, as well known, it is soluble only at acidic pH and its resulting acidic solutions are not recommended for lung administration. As expected, synthesized trimethyl chitosan chloride was more soluble in water [26] and, therefore, suitable for preparing TMC-glycerosomes. Rifampicin (10 mg/ml) was successfully incorporated in the vesicles by direct hydration of rifampicin and phospholipids with a glycerol/water solution (50%) or with the polymer dispersion in the glycerol/water to obtain HY- and TMC-glycerosomes. Glycerol and hydrophilic polymers were used to stabilize phospholipid vesicles and to make them more suitable for pulmonary delivery. [27,28] Physico-chemical characteristics of polymer-glycerosomes and their *in vitro* performances, especially those of TMC-glycerosomes, were very similar to those of glycerosomes and always more promising than those of drug dispersion. Indeed, vesicles were less toxic and with a greater aptitude to be aerosolized respect to the free drug dispersion. In addition, glycerosomes and polymer-glycerosomes were able to increase the antibacterial activity against *S. aureus*, used as representative airway colonizing pathogen. Probably, this was because the vesicles facilitate the drug interaction with the bacterium surfaces. The *in vitro* MIC predicts bacterial response to specific chemical agent or formulation and allowed us an initial screening among the formulations. An *in vitro* decrease of antibiotic MIC is indicative of a suitable challenge to ameliorate treatment against host bacteria *in vivo*, especially when it is associated to a higher local drug accumulation in target tissue. Actually, HY-glycerosomes provided a lower *in vitro* rifampicin MIC against *S. aureus* in comparison with the free drug as well as the highest *in vivo* drug retention in lungs. In fact, the mean rifampicin concentration in lungs obtained with HY-glycerosomes (8.5 μg/g) was about six fold higher than that provided by TMC-glycerosomes (1.5 μg/g, $p<0.05$) probably due to the better aptitude of these vesicles to be aerosolized and retained in the lung tissue. Taking into account these results, we can hypothesize that the improvement of nebulization aptitude and deposition of rifampicin in the lungs associated to an increased antibacterial activity, can

provide a higher local drug bioavailability and efficacy avoiding drug systemic distribution and its associated adverse effects. Moreover, direct pulmonary administration of rifampicin loaded HY-glycerosomes allowed us to obtain local drug concentrations higher than 5 μg/g up to 24 h with a single administration, which can impair the development of bacterial resistance.

5. Conclusions

The *in vitro* and *in vivo* findings of this study underlines the promising properties of HY-glycerosomes as suitable carriers for rifampicin delivery to the lungs. The association of sodium hyauronate and glycerol to the liposomes led to obtain a suitable rifampicin formulation that was no toxic *in vitro* for A549 cells and had a great activity against *S.aureus*. Additionally, this formulation was easily areosolized and provided a high rifampicin deposition in lungs. Our outcomes confirm that suitable nanotechnology may play an important role in enhancing the local pharmacological activity of rifampicin, reducing the number of daily administrations, possible side effects and improving drug efficacy.

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Figure Captions

Figure 1. ^{1}H-NMR spectrum of the synthesized TMC

Figure 2. Negative electron transmission micrographs of rifampicin loaded glycerosomes (A), HY-glycerosomes (B) and TMC-glycerosomes (C).

Figure 3: In vitro cytotoxic effect of rifampicin in dispersion and loaded in vesicles on A549 cells as a function of drug concentration and incubation time. Mean values of 3 independent samples are reported±standard deviation (bars).

Figure 4. Concentrations of rifampicin in the plasma of rats along 24 h (A) and in different parts of the respiratory tree at 24 h (B) after the intratracheal administration of samples. Mean values are reported ± standard deviations (Bars).

Figure 5. Rifampicin concentrations in the liver and lungs of rats 24 h after the intratracheal administration of the assayed samples. Mean values are reported and bars represent standard deviations. Symbols * and ° indicate values statistically different in comparison with control dispersion and between glycerosomal samples ($p<0.05$).