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Fabrication of polyelectrolyte multilayered vesicles as inhalable dry powder for lung administration of rifampicin

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ABSTRACT

A polyelectrolyte complex based on chitosan and carrageenan was used to coat rifampicin-loaded vesicles and obtain a dry powder for inhalation by spray-drying. The polymer complexation on vesicle surface stabilized them and improved their adhesion on airways and epithelia cells. Uncoated liposomes were small in size, negatively charged and able to incorporate large amounts of rifampicin (70%). Coated vesicles were still able to load adequate amounts of drug (~70%) but the coating process produced larger particles (1 μm) that were positively charged and with a spherical shape. Aerosol performances, evaluated using the next-generation impactor, showed that coated vesicles reached the 50% of fine particle fraction and the smallest mass median aerodynamic diameter (2 μm). Rifampicin-loaded uncoated and coated vesicles slowly reduced the A549 cell viability over a 48-h incubation time. Moreover, *in vitro* coated formulations had a strong ability to be easily internalized and to greatly prolong the residence time of their components in A549 cells compared to uncoated liposomes that were rapidly internalized and just as quickly removed.

1. Introduction

Pulmonary drug delivery systems have been increasingly studied and developed thanks to their suitability to quickly allow high local or systemic drug bioavailability avoiding the first-pass effect (Mahajan and Gundare, 2013; Mansour et al., 2009; Mura et al., 2011b). Lungs may be used as a route of administration for local pulmonary diseases such as asthma, bronchitis and emphysema and other severe diseases that compromise the lungs functionality, including cystic fibrosis, tuberculosis, pulmonary vascular disorders and interstitial lung disorders, or for systemic diseases (Nahar et al., 2013). In particular, inhalable drug delivery systems represent a viable alternative to administer local medicaments to different sites of the respiratory tree (Kawashima et al., 1998). To reach therapeutic efficacy, inhalable drug formulations must efficiently arrive and deposit in the airways and their aerodynamic size and aerosolization properties are crucial parameters to control the drug deposition in the desired region of the lungs. Moreover, the lung retention of formulations and their ability to cross lung barriers are additional advantages to improve therapeutic efficacy, which can be limited by the mucociliary clearance (Mansour et al., 2009; Pison et al., 2006).

Rifampicin (3-(4-methyl-l-piperazinyl-irninomethyl) rifampicin) is one of the most potent and broad-spectrum antibiotics, frequently used for different pulmonary diseases (Suarez et al., 2001; Sung et al., 2009). Additionally, it represents the first-choice antibiotic when drug resistance develops during chronic treatments of different diseases such as tuberculosis or cystic fibrosis. Unfortunately, this drug is poorly adsorbed after oral administration and doses required to obtain the therapeutic effects are generally too high and associated with various side effects. Administration of rifampicin to the lungs by dry powder formulations may improve its local bioavailability and efficacy, increasing the patient compliance (Coowanitwong et al., 2008; Sung et al., 2009). Among different colloidal systems, rifampicin-loaded liposomes have been widely studied as an inhalable drug delivery system able to effectively improve their pulmonary distribution and bioavailability (Manca et al., 2008, 2012; Zaru et al., 2007, 2009b). Nevertheless, the use of liposomes is limited by their stability in solution and biological environment, and equally significant is their poor stability during the

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spray-drying process, which becomes essential for obtaining a suitable inhalable dry powder.

In the present work, to stabilize rifampicin-loaded liposomes and to obtain a suitable inhalable dry powder, vesicles were coated layer-by-layer with chitosan and carrageenan, two marine-derived polymers. Chitosan is a polycationic polysaccharide largely used in pharmaceutical formulations because of its high biocompatibility and biodegradability (De Souza et al., 2009; Halim et al., 2012; Mura et al., 2011a; Rodrigues et al., 2012b). Chitosan-coated liposomes have already been shown to have a prolonged residence time in the pulmonary tract in comparison to uncoated liposomes (Manca et al., 2012; Moretton et al., 2013; Zaru et al., 2009a). Carrageenan is another polysaccharide composed of galactose and anhydrogalactose units, linked by glycosidic bonds (Lim et al., 2010). Due to its half-ester sulphate moieties, carrageenan displays a strong ionic nature and a good gel-forming ability (Campo et al., 2009; Mano et al., 2007; Thành et al., 2002). Thanks to their excellent properties such as thickening, gelling and stabilizing additive, it is widely used at low concentrations in pharmaceutical formulations. Indeed, at low concentrations, carrageenan can control and regulate pulmonary absorption of drugs, while at high concentrations after direct injection into the pleural cavity it causes the formation of oedema, tissue injury and neutrophil infiltration in lungs (Rodrigues et al., 2012a; Yamada et al., 2005). Chitosan and carrageenan on the vesicle surface may form a polyelectrolyte complex polymer able to protect liposomes and improve their adhesion on epithelia of airways and lungs. Rifampicin was incorporated into liposomes made with a mixture of soy phosphatidylcholine, hydrogenated soy phosphatidylcholine and cholesterol. Vesicles were coated layer-by-layer with chitosan and carrageenan, using initially chitosan followed by carrageenan or, alternatively, carrageenan followed by chitosan, achieving vesicles with different surface charge features. To prepare suitable inhalable dried powders with good aerodynamic properties, calcium carbonate and lactose were added to the water phase of uncoated and coated liposomes and subsequently vesicle dispersions were spray-dried.

Rifampicin-loaded uncoated and coated vesicles were fully characterized for size and size distribution, surface charge and morphology. Differential scanning calorimetry and X-ray diffraction techniques were used to evaluate the molecular state and interactions between polymers, phospholipids and drug. Aerosol performances of powders were evaluated using the next-generation impactor and the Turbospin® as the inhaler device. Finally, the effect of the formulations on cell viability and vesicle uptake on human alveolar cells (A549) was studied using the MTT test and confocal microscopy, respectively.

2. Material and methods

2.1. Material

Soy phosphatidylcholine (Phospholipon® 50, P50, with 45% phosphatidylcholine and 10–18% phosphatidylethanolamine, 3% triglycerides, 0.25% D,L-α-tocopherol, and ≈37% fatty acids) and hydrogenated soy phosphatidylcholine (Phospholipon® 90H, P90H) were kindly supplied by AVG S.r.l. (Garbagnate Milanese, Italy) and Lipoid GmbH (Ludwigshafen, Germany). Rifampicin (RFP), chitosan (CH) with low molecular weight (50,000–190,000 and 75–85% deacetylated), k-carrageenan (CR), cholesterol, lactose, calcium carbonate and all the other products were of analytical grade and were purchased from Sigma–Aldrich (Milan, Italy). Size 2 gelatin capsules were purchased from Farmauno (Castelnuovo di Porto, Italy). The Turbospin® was kindly provided by PH&T S.p.A. (Milan, Italy). All the cell culture reagents were purchased from Life Technologies Europe (Monza, Italy).

2.2. Vesicle preparation

Rifampicin-loaded liposomes were prepared using a mixture of P50, P90H and cholesterol. All the lipid components were weighed in a glass flask, hydrated with dextrose water solution and finally sonicated (5 s on and 2 s off, 30 cycles; 15 μm of probe amplitude) with a high intensity ultrasonic disintegrator (Soniprep 150, MSECrowley, London, United Kingdom) (Castangia et al., 2013). Liposomes were diluted in water (1:3) to obtain the final dispersion concentration of uncoated vesicles (Table 1). To prepare coated vesicles, chitosan (0.5% w/v) was dissolved under stirring in acetic acid (0.1% v/v) aqueous solution and carrageenan (0.1% w/v) was dissolved in distilled water. In a typical preparation, rifampicin-loaded liposomes (25 ml) were slowly added to the chitosan dispersion (25 ml), maintained under stirring, and then the carrageenan dispersion (25 ml) was added; alternatively, liposomes (25 ml) were slowly added to the carrageenan solution (25 ml), maintained under stirring, and afterward the chitosan dispersion (25 ml) was added to obtain the microcomplex (Table 1). Vesicle dispersions were dialyzed against water to eliminate the unentrapped drug. Coated vesicles were centrifuged at 30,000 rpm for 30 min to eliminate excess of polymeric material. The pellet was resuspended in fresh water, while the supernatant was lyophilized and the dry powder was weighed to quantify the amount of unaggregated polymers. The coating polymer efficiency was expressed as the percentage (CP%) of unaggregated polymer versus the amount initially used.

Finally, lactose and calcium carbonate were added to uncoated and coated liposomes, and dispersions were spray dried using a Minispray Dryer (Büchi 190, Switzerland) with a standard 0.7-mm nozzle to produce dried powders. The inlet temperature, spray flow and compressed spray air flow (represented as the volume of the air input) were set at 140 °C, 6 ml/min and 10 ml/min, respectively. Spray-dried formulations were stored at 5 ± 1 °C before nebulization.

2.3. Vesicle characterization

Formation and morphology of formulations were evaluated using a Hitachi S4100, scanning electron microscope (SEM). Size distribution (average diameter and polydispersity index, P.I.) and zeta potential of the samples were measured using a Zetasizer nano (Malvern Instrument, London, United Kingdom) after their dilution with distilled water. Samples were backscattered by a helium–neon laser (633 nm) at an angle of 173° and at a constant temperature of 25 °C. Zeta potential was estimated by means of the M3-PALS (phase analysis light scattering) technique, which measures the particle electrophoretic mobility in a temperature-controlled cell.

Table 1
Composition of rifampicin-loaded uncoated and coated liposome dispersions.

	RFP (% w/w)	P90H (% w/w)	P50 (% w/w)	Chol (% w/w)	CH (% w/w)	CR (% w/w)	$CaCO_3$ (% w/w)	Lattosio (% w/w)
Liposomes	3.2	9.6	9.6	3.2	–		1.6	64
CR/CH liposomes	3.2	9.6	9.6	3.2	1.6	0.3	1.6	64
CH/CR liposomes	3.2	9.6	9.6	3.2	1.6	0.3	1.6	64