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Preparation of drug-loaded small unilamellar liposomes and evaluation of their potential for the treatment of chronic respiratory diseases

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ABSTRACT

The aim of the present investigation was to evaluate the influence of liposome formulation on the ability of vesicles to penetrate a pathological mucus model obtained from COPD affected patients in order to assess the potential of such vesicles for the treatment of chronic respiratory diseases by inhalation. Therefore, Small Unilamellar Liposomes (PLAIN-LIPOSOMEs), Pluronic® F127-surface modified liposomes (PF-LIPOSOMEs) and PEG 2000PE-surface modified liposomes (PEG-LIPOSOMEs) were prepared using the micelle-to-vesicle transition (MVT) method and beclomethasone dipropionate (BDP) as model drug. The obtained liposomes showed diameters in the range of 40–65 nm, PDI values between 0.25 and 0.30 and surface electric charge essentially close to zero. The encapsulation efficiency was found to be dependent on the BDP/lipid ratio used and, furthermore, BDP-loaded liposomes were stable in size both at 37 °C and at 4 °C. All liposomes were not cytotoxic on H441 cell line as assessed by the MTT assay. The liposome uptake was evaluated through a cytofluorimetric assay that showed a non-significant reduction in the internalization of PEG-LIPOSOMEs as compared with PLAIN-LIPOSOMEs. The penetration studies of mucus from COPD patients showed that the PEG-LIPOSOMEs were the most mucus-penetrating vesicles after 27 h. In addition, PEG- and PF-LIPOSOMEs did not cause any effect on bronchoalveolar lavage fluid proteins after aerosol administration in the mouse. The results highlight that PEG-LIPOSOMEs show the most interesting features in terms of penetration through the pathologic sputum, uptake by airway epithelial cells and safety profile.

1. Introduction

Pulmonary drug delivery constitutes the primary option for the treatment of airways diseases such as asthma, bronchitis, chronic obstructive pulmonary diseases (COPD) and cystic fibrosis (CF), since it allows drug administration directly to their site of action (Groneberg et al., 2003). With the exclusion of the alveolar region, a mucus gel covers the airway epithelium, where it plays several important functions. In the healthy lungs, the airway mucus protects the underlying epithelium entrapping irritant substances and microorganisms and contributes to their removal by mucociliary clearance (Knowles and Boucher, 2002; Randell et al., 2006). The two primary determinants that impede inhaled particles to overcome the mucus barrier and become trapped in airway mucus are the steric hindrance and the adhesive trapping (due to electrostatic and hydrophobic interactions) (Duncan et al., 2016). The size of healthy airway mucus pores has been found to be in the range of 100–500 nm, and particles as large as 200 nm could penetrate through it (Schuster et al., 2013). Pathological respiratory mucus (*i.e.*, sputum) shows similar dimensions of pore size, *i.e.* 140 ± 50 nm (range 60–300 nm) in CF (Suk et al., 2009) and in

Abbreviations: BALF, bronchoalveolar lavage fluid; BDP, beclomethasone dipropionate; CF, cystic fibrosis; COPD, chronic obstructive pulmonary disease; FACS, fluorescence-activated cell sorting; LissRhod PE, 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine-N-(lissaminerhodamine B sulfonyl) ammonium salt; LS100, lipoid S100; MVT, micelle-to-vesicle transition method; NBD-PC, 1-palmitoyl-2-[12-[(7-nitro-21,3-benzoxadiazol-4-yl)amino]dodecanoyl]-sn-glycero-3-phosphocholine; NPs, polymeric nanoparticles; PC, phosphatidylcholine; PCS, photon correlation spectroscopy; PDI, polydispersity Index; PEG, polyethylene glycol; PEG-LIPOSOMEs, PEG 2000PE-surface modified liposomes; PEG-2000-PE, 1,2-dipalmitoyl-sn-glycero-3-phosphoethanolamine-*N*-[methoxy(polyethylene glycol)-2000]; PF-LIPOSOMEs, Pluronic® F127-surface modified liposomes; PLAIN-LIPOSOMEs, surface unmodified liposomes; SEC, size exclusion chromatography; SLF, Simulated Lung Fluid; SUVs, Small Unilamellar Vesicles

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average 150 nm in chronic rhinosinusitis (Lai et al., 2011). However, in pathological respiratory conditions, a hypersecretion of highly viscoelastic airway mucus occurs thus altering its pristine function and worsening the respiratory disease. Particles that strongly interact with physiological and pathological mucus components are trapped within the mucus gel depending on their size and surface chemistry. Entrapped particles will be then removed rapidly by the mucociliary clearance and will not reach the airway epithelium. On the other hand, it is noteworthy that mucociliary clearance is severely reduced in asthma, CF and COPD (Boucher, 2007; Fahy and Dickey, 2010; Munkholm and Mortensen, 2014), allowing particles with the appropriate size and hydrophobic surface to penetrate the mucus.

In light of the above, a promising approach to overcome these shortcomings seems to be the use of nano-sized delivery systems because they may allow sustained drug release, reduction of side effects and increased patient compliance. Moreover, the nano-sized delivery systems may protect the active principle from degradation processes and can be appropriately tailored for targeted drug delivery (Andrade et al., 2010; Forier et al., 2014; Rytting et al., 2008). Among the nanocarriers used for the pulmonary delivery, polymeric nanoparticles (NPs) are the most investigated (Lai et al., 2007; Lai et al., 2009; Sanders et al., 2000; Suk et al., 2009). It has been found that polymeric NPs modified with either amphiphilic or hydrophilic nonionic polymers, such as Pluronic® F127 (PF127) or PEG, are able to pass through the mucus barrier and to reach the underlying epithelial cells (Dünnhaupt et al., 2015). Besides polymeric NPs, most promising vehicles for pulmonary administration are lipid-based nanocarriers, including liposomes and solid lipid nanoparticles (SLNs), due to their good biocompatibility and biodegradability, formulation stability, reduced side effects and sustained drug release (Mehnert and Mäder, 2001; Weber et al., 2014; Willis et al., 2012). Interestingly, ultra-small (< 100 nm) SLNs have recently been prepared and their mucus penetration and usefulness in pulmonary delivery of anti-infective agents has been demonstrated (Nafee et al., 2014). Concerning liposomes, despite most attention has been paid in their use as drug carriers for chronic lung infection therapy (Willis et al., 2012), surprisingly efforts to develop vesicles able to avoid both the adhesion to mucin network and the steric inhibition by the dense fiber are still lacking (Li et al., 2011). In this regard, although some studies have been carried out on mucus-penetrating liposomes for oral delivery or for vaginal mucosa imaging (Bourganis et al., 2015; Forier et al., 2014; Hadinoto and Cheow, 2014), to the best of our knowledge, similar investigations on liposomes for pulmonary administration have not been previously described. Moreover, only few studies have evaluated whether modified liposomes (e.g. by pegylation) given by nebulization are more useful for improving lung deposition and drug delivery (Konduri et al., 2003; Konduri et al., 2005).

Therefore, as a novelty in this field, the present study was undertaken to assess whether small liposomes (< 100 nm) are capable of passing through the dense fiber network of the respiratory sputum and evaluate so their potential for the treatment of chronic respiratory diseases by inhalation, in particular COPD patients. It is noteworthy that the herein followed approach of using nano-liposomes for inhalation in the presence of pathological mucus, but not artificial mucus nor mucus obtained from healthy donors, is relevant from a clinical point of view. In this light, Small Unilamellar Vesicles (SUVs) with and without a polymeric surface modifier such as PF127 or PEG lipids (PEG 2000PE) were prepared for pulmonary delivery of the glucocorticoid beclomethasone dipropionate (BDP) selected as anti-inflammatory model drug (Craparo et al., 2016). It is well known that the typical size of liposomes is > 150 nm while, the preparation of small vesicles (< 100 nm) requires appropriate procedures (Bozzuto and Molinari, 2015; Jaafar-Maalej et al., 2010; Willis et al., 2012). Thus, the required SUVs were prepared using the so-called micelle-to-vesicle transition (MVT) method (De Leo et al., 2014). The influence of process parameters (i.e., phospholipid concentration, PF127, PEG 2000PE and BDP content) on liposome characteristics (size, zeta-potential and morphology) was investigated. The *in vitro* mucus-penetrating properties of these liposomes were investigated together with their cytotoxicity and uptake by pulmonary cells. Moreover, their *in vivo* biocompatibility was also assessed.

2. Materials and methods

2.1. Materials

All chemicals were purchased of the highest purity available and were used without further purification. Ethanol, methanol, Sephadex G-50 medium, cholesterol, beclomethasone dipropionate (BDP), the reagent grade salts for the 50 mM K-phosphate, 100 mM KCl (pH 7.0) buffer solutions and sodium cholate were purchased from Sigma-Aldrich. 1,2-Dipalmitoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy(polyethylene glycol)-2000] (PEG-2000-PE), 1-palmitoyl-2-[12-[(7-nitro-2–1,3-benzoxadiazol-4-yl)amino]dodecanoyl]-sn-glycero-3-phosphocholine (NBD-PC) and 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine-N-(lissaminerhodamine B sulfonyl) ammonium salt (LissRhod PE) were purchased from Avanti Polar Lipids. Lipoid S100 (LS100) was from Lipoid (Lipoid GmbH, Germany). According to the manufacturer's instructions, LS100 is constituted by approximately 100% soybean phosphatidylcholine (PC). Pluronic F127® [poly(ethylene oxide) – poly(propylene oxide) – poly (ethylene oxide) (PEO-PPO-PEO) triblock copolymer, PF127] was a kind gift of BASF (Ludwigshafen, Germany). Sterile filters of cellulose acetate of 0.2 μm were from Advantec. All aqueous solutions were prepared by using water obtained from a Milli-Q gradient A-10 system (Millipore, 18.2 MΩ cm, organic carbon content ≤ 4 μg/L).

2.2. Liposome preparation and characterization

Liposomes were prepared by the MVT method, consisting in the detergent removal from phospholipid/detergent mixed micelles to induce liposome formation, as previously described (De Leo et al., 2014). Briefly, an adequate amount of lipids (usually 10 mg) was dissolved in ethanol and dried with a gentle nitrogen flux to form a homogeneous film on the walls of a conical glass tube. Solvent removal was completed under vacuum conditions (24 h) by a no-oil pump operating at 1 mBar. After that, 0.5 mL of 4% sodium cholate in 50 mM K-phosphate/100 mM KCl (pH 7.0) were added to the dry lipid film and then sonicated (20 shots with a Branson Sonicator 250) to form a clear, translucent mixed micelle solution. The latter was loaded onto a glass column (20 × 1 cm) packed with G-50 Sephadex Medium equilibrated with 50 mM K-phosphate/100 mM KCl (pH 7.0) for detergent removal by size exclusion chromatography (SEC). Liposomes eluted in a 1 mL fraction after a void volume of about 1.5 mL (De Leo et al., 2017a). For surface modified liposomes, 5% w/w of PEG-2000-PE or PF127 was added to the lipid blend before the formation of dry films. For BDP loaded liposomes, an appropriated amount of drug (generally 10 μg per 1 mg total lipids) was added to the lipid blend. Fluorescently labelled liposomes were prepared by adding to the lipid blend either 0.4% of NBD-PC or 0.4% of LissRhod PE for green and red-labelled vesicles, respectively. Liposomes for the *in vitro* and *in vivo* assays were prepared under sterile conditions.

The particle size and polydispersity index (PDI) of unloaded and BDP-loaded liposomes were determined in double distilled water by photon correlation spectroscopy (PCS) using a Zetasizer Nano ZS (ZEN 3600, Malvern, UK). Liposome size distributions were referred as number-based size distribution. The determination of the zeta-potential was performed using laser Doppler anemometry (Zetasizer Nano ZS, ZEN 3600, Malvern, UK) after dilution in KCl (1 mM, pH 7.0) (Mattioli-Belmonte et al., 2014). The liposome morphology was assessed by Transmission electron microscopy (TEM). Briefly, TEM investigation was performed with a JEOL 1011 operated at 100 kV. Samples were prepared via drop casting of liposome suspension in Milli-Q water onto

carbon-coated copper grids. After 2 min, the excess suspension was removed and the wet grid was let to dry overnight. The films were positively stained with 2% w/w phosphotungstic acid solution.

2.3. High-performance liquid chromatography (HPLC) analyses of the encapsulated BDP

The actual amount of BDP encapsulated into BDP-loaded liposomes was determined by HPLC after its extraction from aqueous liposome dispersions with a 1:1:1 ethanol/*n*-hexane/diethyl-ether mixture (Mastrogiacomo et al., 2015). Briefly, 1 mL of aqueous sample was added to 2 mL of extraction mixture and mixed vigorously. After phase separation, a 1.1 mL of organic solution (upper phase), containing all BDP and hydrophobic components, was collected. The extraction of BDP was found quantitative after three washing of liposome suspension with the organic mixture. HPLC analyses of BDP were performed with a Waters (Waters Corp., Milford, MA) Model 600 pump equipped with a Waters 2996 photodiode array detector, a 10 µL injection loop autosampler (Waters 717 plus), and processed by Empower™. Software Build. For analysis, Zorbax Eclipse (25 cm × 4.6 mm; particle size of 5 µm, Agilent) column was eluted with 80:20 (v:v) methanol: water in isocratic mode. The flow rate of 1.2 mL/min was maintained and the column effluent was monitored continuously at 254 nm. The quantification of the compound was carried out by measuring the peak areas in relation to those of standards chromatographed under the same conditions. Standard calibration curves were prepared at 254 nm wavelength using the eluent above mentioned as the solvent and were linear ($r^2 > 0.999$) over the range of tested concentrations (from 1.9×10^{-9} M to 1.9×10^{-12} M). The retention time of BDP was 9.0 min.

Drug encapsulation efficiency (EE) was calculated by using the following equation:

$$EE(\%) = (Q_{encapsulated}/Q_{initial}) \times 100$$

where $Q_{initial}$ is the amount of initially added BDP per milliliter of liposome suspension, and $Q_{encapsulated}$ is the amount of entrapped drug per milliliter of liposome suspension, determined by HPLC.

2.4. In vitro release of BDP from liposomes

The *in vitro* BDP release kinetics from liposomes was evaluated according to our previously published protocol (Trapani et al., 2013). Briefly, BDP-loaded liposomes (PLAIN-LIPOSOMEs, PEG-LIPOSOMEs and PF-LIPOSOMEs) were freshly prepared and each test sample was provided as a liposomal suspension of a volume of 50 µL corresponding to 2–2.8 mg of pure BDP.

Such donor was suspended in a mixture of 12.7 mL containing Simulated Lung Fluid (SLF, pH 7.4) and EtOH (99/1,% v/v). The composition of SLF is reported in our previous work (Trapani et al., 2013). The dispersion was divided into 7 Eppendorf tubes, each one containing 1.82 mL of the medium. The tubes were then kept in a shaker at 37 °C at 100 rpm up to 48 h. At appropriate time intervals (0, 1, 3, 5, 16, 24 and 48 h), these tubes were taken out from shaker and ultracentrifuged at 4 °C at 100 k × g. The supernatant was analyzed for the BDP content according the HPLC protocol above reported.

2.5. Stability of BDP loaded liposomes

The stability of BDP-loaded liposome formulations (*i.e.*, PLAIN-LIPOSOMEs, PEG-LIPOSOMEs and PF-LIPOSOMEs) was investigated by their incubation at two different temperatures, 37 °C (100 rpm) and 4 °C (with no stirring) respectively. In particular, the exposure at 37 °C was followed up to 48 h whereas the exposure at 4 °C was up to 4 weeks. The particle size was measured at different time intervals after a 1:3 dilution in double distilled water by PCS. Each experiment was performed in triplicate.

2.6. Sputum penetration studies

Spontaneous sputum samples were collected from a total of 8 COPD affected adult patients, enrolled at the Respiratory Diseases Clinic (Ospedali Riuniti di Foggia) after they signed informed consent. The work described has been carried out in accordance with The Code of Ethics of the World Medical Association (Declaration of Helsinki). Each sample was collected into a clear plastic Petri dish and the saliva pulled out of sputum by forceps. Two to three samples were acquired from the weekly COPD outpatient clinic and pooled together to minimize patient-to-patient variation, and studied on the same day. Sputum diffusion studies were performed according to previously published works (Friedl et al., 2013; Karamanidou et al., 2015). In brief, 24-well plates with transwell inserts (Transwell®, Corning, pore size 0.4 µm) occupying a surface of 33.6 mm^2 and covered by 100 µL of sputum were used. The sputum was left to settle on a shaking board (300 rpm) at 37 °C for 10 min. Afterwards, 600 µL of phosphate buffer (PBS) (25 mM, pH 6.5) was added to the basolateral (acceptor) chamber and the apical (donor) chamber was filled up by 200 µL of LissRhod PE-loaded liposomes dispersions (containing 10 mg of lipids per mL) in the same buffer. The plates were incubated at 37 °C on a shaking board at 100 rpm. For a 27-h incubation period, samples of 100 µL were withdrawn from the basolateral (acceptor) chamber at different intervals (2, 4, 6, 21 and 27 h) and replaced by the same volume of buffer, preheated at 37 °C. The fluorescence intensity of each collected sample was measured in 96-well plates at an excitation wavelength of 535 nm and an emission wavelength of 595 nm using a fluorescence microplate reader (Filter Max F5, Molecular Device). For each type of liposome, the diffusion across the mucus, at a defined time point, was determined by calculating the "permeation percentage". This parameter was obtained by dividing the fluorescence of each sample in presence of mucus by the fluorescence of the sample in absence of mucus. The buffer without liposomes was stratified onto mucus and served as a blank for measuring the background level of fluorescence.

2.7. Cell cultures and cytotoxicity

H441 cells were cultured in RPMI 1640 medium containing 5% FBS, 4.5 g/L glucose, 100 U/mL penicillin and 100 µg/mL streptomycin. H441 cells were seeded onto 96-wells plates at a density of 3×10^4 cells/well and allowed to attach for 24 h. Liposomal formulations were prepared in RPMI containing 10% FCS (Fetal Calf Serum) at the desired final concentrations of BDP (in a 0.04–4 µM range, corresponding to 0.02–2 mg/L lipid) and added to each well for 24 h. A stock solution of MTT (Sigma) in phosphate buffered saline (PBS) (5 mg/mL) was added to each well reaching a final concentration of 0.5 mg/mL (in 1000 µL of complete medium). After 4 h the formazan crystals were dissolved in a 100 µL of DMSO and measured spectrophotometrically by an ELISA reader (Filter Max F5, Molecular Device) at a wavelength of 570 nm with a reference wavelength of 630 nm. The relative viability was calculated with respect to control wells containing mock cells, *i.e.* cells treated with 150 mM NaCl (considered as 100%). 10% SDS-treated cells were used as positive control.

2.8. Uptake of liposome preparations by H441 cells

6×10^4 cells were seeded onto 24-well plates and incubated with LissRhod PE-labelled liposome (ranging between 0.02 and 2 mg/mL of lipid content) for 24 h at 37 °C. The analysis of uptake was carried out as previously described (Di Gioia et al., 2015). Briefly, cells were halved in two samples, one treated and the other not treated with trypan blue 0.04% in PBS in order to quench extracellular fluorescence and they were analyzed by fluorescence-activated cell sorting (FACS) with the "FlowSight1 Flow Cytometer" (Amnis; Merck Millipore). The percentage of positive cells was determined setting the gating on 99% of an untreated control population of cells and by subtracting their

fluorescence. Ten thousand cells were examined in each analysis. The percentage of positive cells was detected by exciting samples with a 557 nm laser light and recording the emission at 576 nm.

2.9. In vivo studies

All the *in vivo* procedures have been carried out according with the EU Directive 2010/63/EU for animal experiments and under approval of the local ethics committee. The *in vivo* work was performed also in compliance with the Code of Ethics of the World Medical Association (Declaration of Helsinki). The aerosol administration was performed as previously described (Trapani et al., 2013). Liposomal formulations were prepared in 150 µL of sterile PBS and were administered to Swiss mice (25 g, Charles River, Calco, Italy) using a 245 MicroSprayer™ aerosoliser (IA–1C; Penn Century, Philadelphia, PA, USA) suitable for mice, attached to a high-pressure syringe (FMJ – 250; Penn Century). The mice were anaesthetized by an intraperitoneal injection of 0.5 mg/g body weight Avertin (2,2,2 – Tribromoethanol; Sigma Aldrich, Germany) and were suspended at a 45° angle by the upper teeth. The light source's (lamp type FLQ85E; Helmut Hund, Germany) flexible fiber-optics arm was adjusted to provide optimal illumination of the trachea. A small spatula was used to open the lower jaw of the mouse and blunted forceps were used to help displace the tongue for maximal oropharyngeal exposure. After a clear view of the trachea was obtained by an otoscope, the MicroSprayer tip was endotracheally inserted until it reached the carina (the first bronchial bifurcation) and the liposomal preparation was sprayed. The tip was immediately withdrawn and the mouse was taken off the support. The collection of the bronchoalveolar lavage fluid (BALF) was performed at 24-h post-dosing as previously described (Trotta et al., 2013). The protein concentration in the BALF was assayed by the Bradford assay using a bovine serum albumin curve and was expressed as µg/mL BALF.

2.10. Statistical analysis

Data from different experimental groups were compared by a one-way analysis of variance (ANOVA) with Tukey's multiple comparison test or unpaired Student's *t*-test by using GraphPad Prism v. 5.00 computer program (GraphPad Software, Inc. CA, USA). Differences were considered significant at a confidence level of 95% ($p < .05$).

3. Results

3.1. Preparation and characterization of PLAIN-LIPOSOMEs, PEG-LIPOSOMEs and PF-LIPOSOMEs

PLAIN-, PEG- and PF-LIPOSOMEs were successfully prepared by MVT method (De Leo et al., 2014) as described in the Materials and Methods section. Preliminary trials were devoted to elucidate the influence of the process parameters (*i.e.* phospholipid concentration, excipients and drug content) on liposome characteristics. Table 1 shows the particle size of liposomes when the cholesterol content and the sodium cholate detergent percentages were fixed at 25% w/w_{lipids} and 4% w/v respectively, while the concentration of LS100 ranged from 8 to 16 mg/mL. The observed PDI values for all the vesicles prepared were in the range 0.25–0.30 suggesting a slightly broad size distribution (data not shown). Table 1 also shows the effect of the PEG-2000PE and PF127 at the percentage of 2% and 5% w/w_{lipids} on the vesicle size. The tested amount of lipids and of polymeric coatings showed a negligible effect on the size of the liposomes. However, a maximum lipid concentration of 10 mg/mL ensured the best condition for the liposome preparation by the MVT method. Indeed, higher lipid concentrations slowed down the detergent removal step by size exclusion chromatography (SEC) (see Materials and Methods section). Moreover, a 5% w/w_{lipids} percentage of polymeric excipients was chosen to ensure a good surface vesicle coverage. Therefore, these conditions were adopted in the reported subsequent experiments.

Table 1
Dependence of the liposome mean size on the lipid and modifier content.

Liposome	Lipid content (mg/mL)	Modifier content (% w/w)	Mean Diameter[a] (nm)
PLAIN-LIPOSOMEs	8		40 ± 6
PLAIN-LIPOSOMEs	10		54 ± 8
PLAIN-LIPOSOMEs	12		50 ± 8
PLAIN-LIPOSOMEs	16		49 ± 7
PEG-LIPOSOMEs	10	2	55 ± 9
PEG-LIPOSOMEs	10	5	64 ± 10
PF-LIPOSOMEs	10	2	51 ± 8
PF-LIPOSOMEs	10	5	65 ± 10

[a] Values are expressed as mean ± standard deviation (n = 3).

Table 2
Dependence of EE on the BDP/lipid ratio.

Liposome	BDP/Lipids mass ratio (µg/mg)	EE (%)
PLAIN-LIPOSOMEs	5	100 ± 2
PLAIN-LIPOSOMEs	10	100 ± 3
PLAIN-LIPOSOMEs	20	73 ± 5
PLAIN-LIPOSOMEs	30	66 ± 7
PLAIN-LIPOSOMEs	40	43 ± 5

The lipid content for all samples displayed in Table is 10 mg/mL. Encapsulation efficiencies (EE) are expressed as mean values ± standard deviations (n = 3).

The optimal ratio between the amount of drug and lipids leading to the best encapsulation efficiency (EE) was also determined. The EE of PLAIN BDP-loaded LIPOSOMEs was found to be dependent on the drug/lipid ratio used, resulting quantitative for a maximum BDP content of 10 µg/mg of total lipids and progressively decreasing at higher ratios (Table 2). Similar results were obtained for PEG- and PF-LIPOSOMEs (data not shown).

Table 3 shows the mean size and the zeta-potential related to the three types of liposomes selected for this study: *i*) PLAIN LIPOSOMEs, 10 mg/mL of lipids; *ii*) PEG-LIPOSOMEs, 10 mg/mL of lipids and 5% (w/w_{lipids}) of PEG-2000PE; *iii*) PF-LIPOSOMEs, 10 mg/mL of lipids and 5% (w/w_{lipids}) of PF127. A schematic representation of these three types of liposomes is shown in Fig. 1. In addition, Table 3 shows EE and the drug influence on the parameters listed above when the BDP content was set at 10 µg/mg of lipids

Liposome morphology was assessed by TEM. According to the acquired images (Fig. 2), all the examined vesicles showed regular spherical shape confirming that neither the BDP nor the surface modifier-addition significantly affected the liposomal morphology. As expected, the average diameter of the vesicles, as estimated by TEM, was smaller than that obtained by PCS measurement, as this latter

Table 3
Mean size, zeta-potential, and encapsulation efficiency (EE) of liposomes.

Liposome	BDP content (µg/mL)	Mean Diameter[a] (nm)	Zeta-potential[b] (mV)	EE[b] (%)
PLAIN-LIPOSOMEs	—	54 ± 8	−1.7 ± 0.1	—
PLAIN-LIPOSOMEs	10	60 ± 9	−0.4 ± 0.3	100 ± 3
PEG-LIPOSOMEs	—	64 ± 10	−2.5 ± 0.3	—
PEG-LIPOSOMEs	10	70 ± 10	−2.4 ± 0.3	83 ± 4
PF-LIPOSOMEs	—	65 ± 10	+0.3 ± 0.2	—
PF-LIPOSOMEs	10	72 ± 11	+0.8 ± 0.3	80 ± 4

The lipid content for all the liposome types displayed in Table is 10 mg/mL. The PEG-2000PE and PF127 percentage is 5% (w/w) for both the modified liposome types.

[a] Mean diameters are expressed as mean ± standard deviation (n = 3).

[b] Zeta-potentials and encapsulation efficiencies are expressed as mean values ± standard deviations (n = 3).

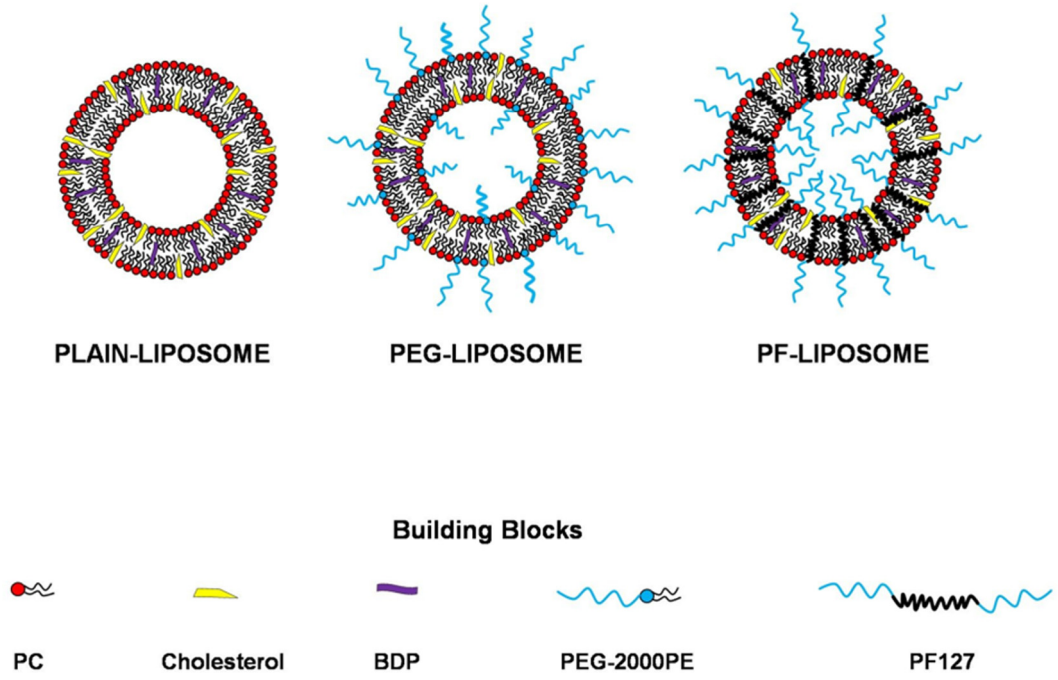

Fig. 1. Schematic representation of the three types of liposomes tested for the evaluation of mucus-penetrating properties. This sketch is merely qualitative. The dimensions of the different components are not to scale.

technique is strongly influenced by the presence of even a small fraction of large particles. Indeed, the intensity of the scattered light is proportional to the sixth power of the particle radius (De Leo et al., 2014).

3.2. *In vitro drug release from liposomes*

The *in vitro* release kinetics of BDP loaded liposomes were determined in SLF/EtOH (99/1,%v/v) mixture without enzymes. The composition of the release medium was designed in order to be mimetic with the pulmonary environment. In order to enhance the BDP release in SLF, a small amount of EtOH was added to the medium, since the BDP is a very low water soluble drug (Trapani et al., 2015; Triolo et al., 2017). As shown in Fig. 3, all liposomes released negligible amounts of BDP within 48 h, with the smallest amount (0.2%) released by PF-LIPOSOMEs, whereas PLAIN- and PEG-LIPOSOMEs released about 1% of the drug. Except for PF-LIPOSOMEs, in the other cases an initial burst effect was evident.

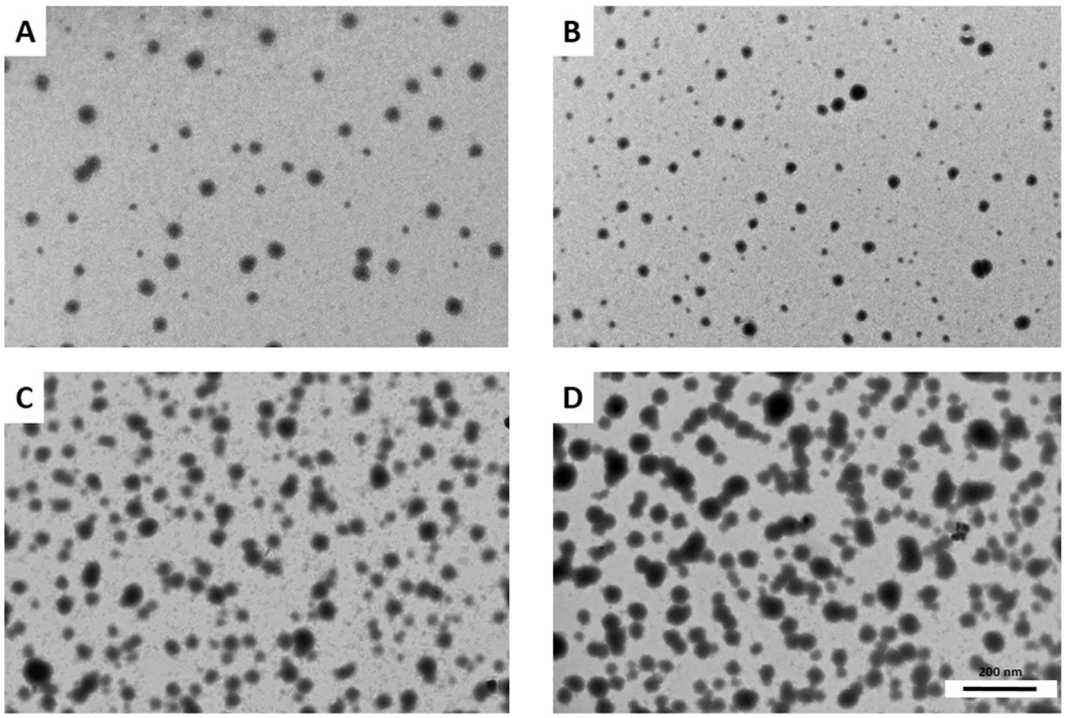

Fig. 2. TEM images of (A) PLAIN-LIPOSOMEs, (B) BDP-loaded PLAIN-LIPOSOMEs, C) BDP-loaded PEG-LIPOSOMEs, D) BDP-loaded PF-LIPOSOMEs. Scale bar: 200 nm.

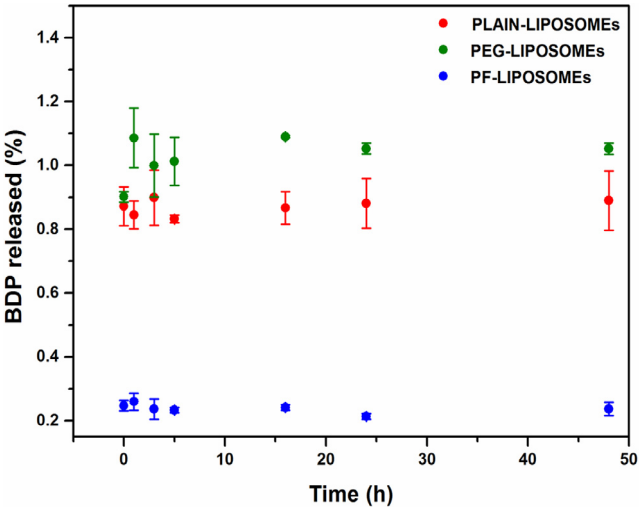

Fig. 3. *In vitro* BDP release from surface unmodified and modified liposomes. The experiments for each type of liposomes were performed in triplicates and results are shown as means ± standard deviations.

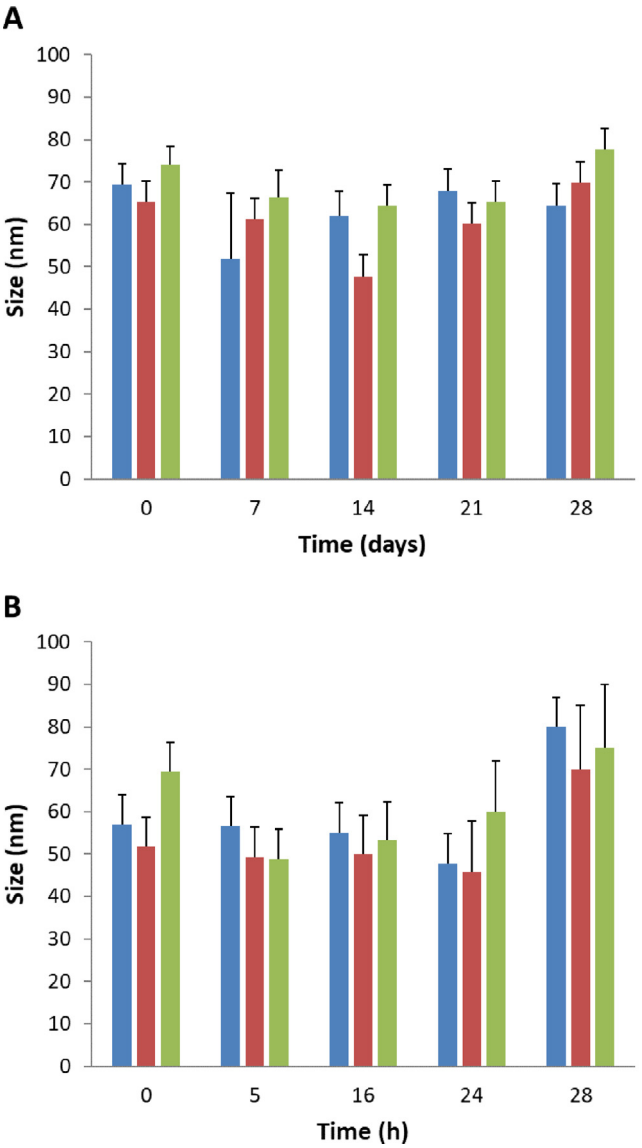

Fig. 4. Stability profiles of BDP loaded liposomes A) at 4 °C (upper panel) and B) at 37 °C (lower panel). PLAIN-LIPOSOMEs (blue bars); PEG-LIPOSOMEs (red bars); PF-LIPOSOMES (green bars). Results are expressed as mean ± standard deviations of three experiments.

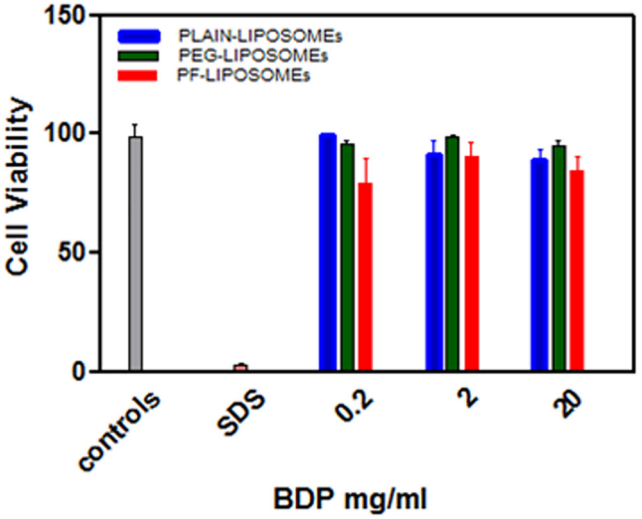

Fig. 5. Cytotoxicity of BDP-loaded liposome formulations. H441 cells were incubated with PLAIN, PEG-, or PF-LIPOSOMEs for 24 h. Each liposome formulation was used at various BDP concentrations. Control indicates untreated cells, while positive controls were obtained with SDS-treated cells. Results are expressed as means ± standard deviations of two experiments, each carried out in six wells.

3.3. Stability studies on BDP loaded liposomes

The stability of the BDP loaded liposomes was studied monitoring their size at 37 °C and 4 °C respectively. The results, reported in Fig. 4, showed that at 37 °C the vesicles were essentially stable for 28 h and that at 4 °C all vesicles resulted quite stable up to four weeks (size < 70 nm).

3.4. In vitro cytotoxicity of liposomes

In order to evaluate the liposome biocompatibility, the three liposome samples were incubated with H441 bronchiolar epithelial cells for 24 h. Cell viability was then assessed by the MTT assay. PLAIN-LIPOSOMEs showed no cytotoxicity at BDP concentrations ranging between 0.2 and 20 mg/mL. In addition, neither PEG-LIPOSOMEs nor PF-LIPOSOMEs were cytotoxic (Fig. 5).

3.5. Sputum permeation studies

Fig. 6 shows the results of sputum penetration studies for the three kinds of liposomes up to 27 h. The permeation percentage (calculated as indicated in Materials and Methods section) was negligible or very low up to 6 h (the last time point commonly considered in mucus penetration studies (Bourganis et al., 2015)). We then extended our observations up to 21 and 27 h. At 21 h, the PEG-LIPOSOMEs displayed a permeation percentage similar to that of the PLAIN-LIPOSOMEs (3.7 ± 1.5% *vs* 2.5 ± 1.3%; mean ± SD), whereas at 27 h they outperformed the PLAIN-LIPOSOMEs (18.8 ± 10.0% *vs* 5.3 ± 2.7%; $p < .05$). The permeation percentage of PF-LIPOSOMEs was lower than the other liposomal formulations also at these extended time points (at 27 h, 1.2 ± 0.8%, $p < .01$ as compared with PEG-LIPOSOMEs).

3.6. Uptake of liposome by H441 cells

To quantitatively evaluate the liposome uptake by the airway epithelial cells, a cytofluorimetric assay based on the quenching of membrane-associated fluorescence (Di Gioia et al., 2015) was performed. After 24 h of cellular incubation with fluorochrome-conjugated liposomes, cells were treated or not with trypan blue before analysis.

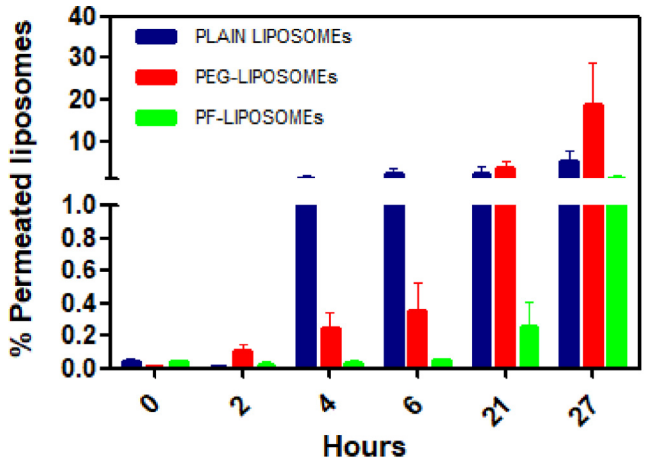

Fig. 6. Penetration of sputum by BDP-loaded liposomes. PLAIN-, PEG- and PF-LIPOSOMEs were tested for the sputum permeation study in a Transwell system. Permeation is reported as percentage of permeation carried out in the absence of sputum. Results are shown as means ± standard deviations (n = 2 in duplicate). $p < .01$ PEG 27 h *vs* PF127 27 h; $p < .05$ PEG 27 h *vs* PLAIN 27 h.

Trypan blue quenches the extracellular fluorescence allowing to determine the intracellular signal only. Therefore, the whole cell-associated fluorescence is detected in cells not treated with trypan blue, while internalized fluorescence is revealed in trypan blue-treated cells. The cellular uptake of liposomes at 0.02 mg/mL of BDP was negligible (data not shown) thereby we focused on the highest concentration tested in the viability assay, i.e. 20 mg/mL of BDP. In general, modified liposomes were taken up at a lower rate than the unmodified ones. Representative images of trypan blue-treated cells in brightfield and in the fluorescent channel are shown in Fig. 7A, while Fig. 7B-D panels depict the difference between mock cells (*i.e.* untreated) and cells incubated with the three different liposomal formulations. Fig. 7E shows that a good uptake (43.6% in the presence of trypan blue) was observed with PLAIN-LIPOSOMEs, whereas lower internalization was obtained with PEG-LIPOSOMEs (22.1%) and PF-LIPOSOMEs (11.9%). However, only PF-LIPOSOME uptake was significantly lower as compared with the PLAIN-LIPOSOMEs.

3.7. *In vivo* toxicity of liposomes

To determine whether liposomes were toxic to the endothelial/alveolar barrier *in vivo*, BDP-loaded liposomes were injected into the trachea of Swiss mice. In order to limit the number of animals involved in the experimentation, only BDP-loaded liposomes were tested, since the empty liposomes showed no cytotoxicity *in vitro*. The final concentration of BDP administered to mice by liposomal formulations was 0.5 mg/kg. This concentration proved to be effective in reducing LPS-induced lung inflammation when dexamethasone palmitate was delivered by mannosylated liposomes (Wijagkanalan et al., 2008). Fig. 8 represents the total protein concentration in BALF, which is an indicator of the endothelial/alveolar barrier integrity. Nebulization of PLAIN-LIPOSOMEs determined a negligible increase in the BALF protein concentration. Interestingly, both PEG- and PF-LIPOSOMEs did not cause any effect on BALF proteins as compared with the controls.

4. Discussion

The aim of the present investigation was to evaluate the influence of liposome formulation on the ability of vesicles to penetrate a pathological mucus model obtained from COPD affected patients in order to assess their potential for the treatment of chronic respiratory diseases by inhalation. Recently, Bernkop-Schnürch and coworkers summarized the successful strategies for mucus layer penetration by several nanocarrier systems (Dünnhaupt et al., 2015). It has been pointed out that

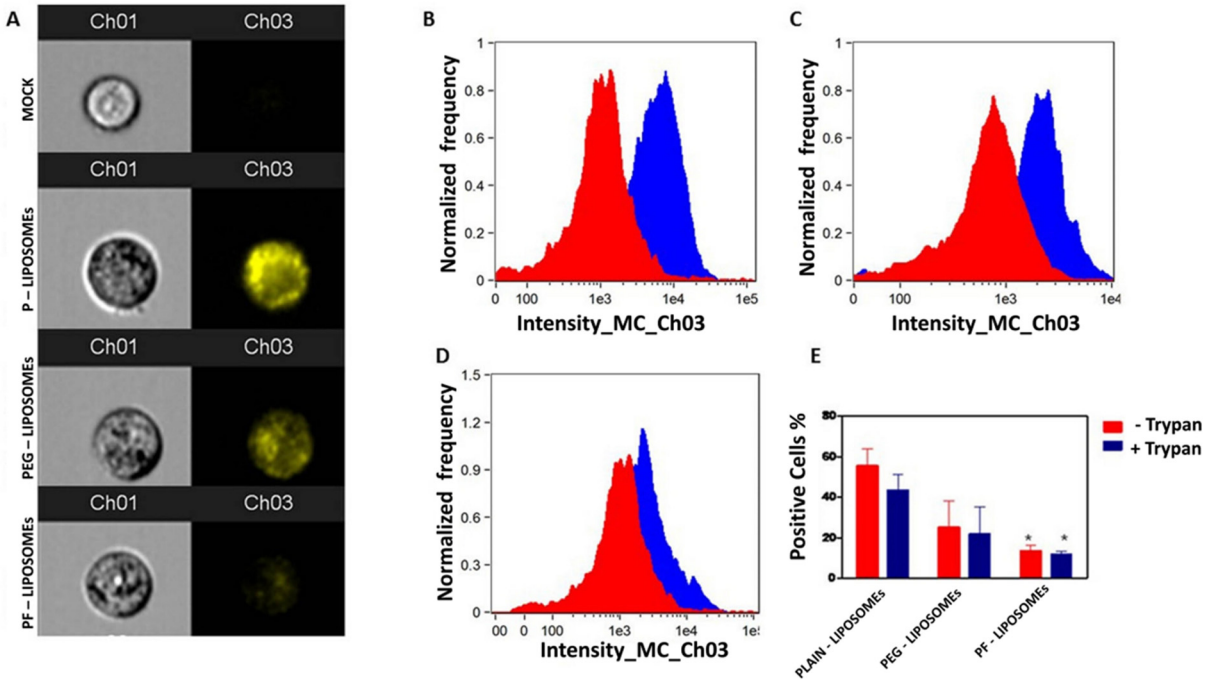

Fig. 7. Uptake of liposomes by H441 cells. Cells were incubated with BDP-loaded liposomes for 24 h and treated or not with trypan blue. (A) Epifluorescence microscopy images showing localization of fluorescent liposomes in H441 cell lines. Ch01 (brightfield channel), Ch02 (red fluorescence channel). (B-D) Cellular uptake of PLAIN-(B), PEG- (C) and PF-LIPOSOMEs (D) by H441 cells by flow cytometric analysis. Red filled histogram in each panel represents untreated cells while blue filled histogram represents liposomes treated cells. Panels (A-D) were generated from data obtained with trypan blue-treated cells. (E) Percentages of positive cells either untreated or treated with trypan blue. Results are shown as means ± standard deviations (n = 2 in triplicate). $^{*}P < .05$ as compared with the PLAIN-LIPOSOME treated cells.

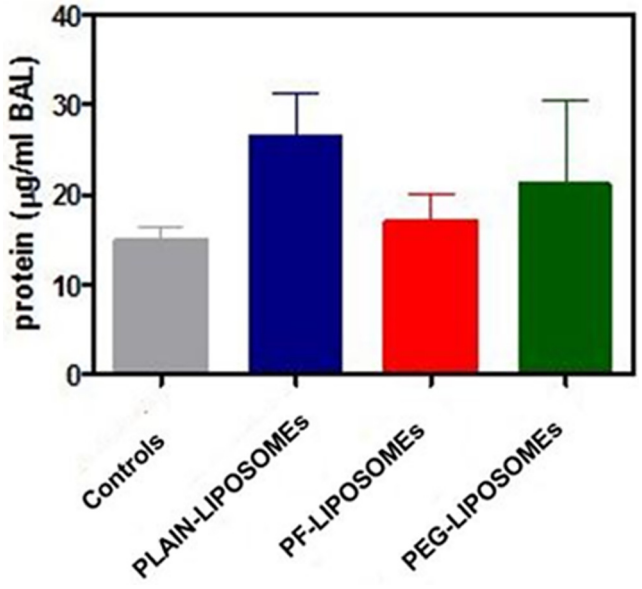

Fig. 8. *In vivo* toxicity. BDP-loaded liposome formulations were administered to the lung of Swiss mice via intratracheal nebulization and the concentration of BALF proteins was assessed 24 h later. Controls were nebulized with saline. Data are expressed as means ± standard errors of the mean of three experiments. In total 3–6 mice per group were used. ANOVA test showed no significant differences of different liposome formulations as compared with controls.

nanocarriers with improved mucus-penetration ability should be modified on their surface either with amphiphilic or hydrophilic polymers with a low hydrogen bonding capability, an approach known as "slippery surface strategy" (Dünnhaupt et al., 2015). In any case, an important role is played by the nanocarrier size which should be low enough to allow their penetration through small mesh size of pathological mucus (Dünnhaupt et al., 2015). To the best of our knowledge, the determination of pore size in COPD sputum has not carried out yet. However, the percentages of nanospheres that diffuse through a layer of CF or COPD sputum with equal thickness (220 µm) are the same (Sanders et al., 2000), indicating a close similarity between CF and COPD sputum in this respect. In the present paper, the slippery technology was applied to liposomes by modifying their surface with PF127 or PEG 2000PE. For a pulmonary delivery purpose, the MTV method employed by us allows to obtain SUVs starting from mixed micelles by detergent removal, a process carried out by size exclusion chromatography that enables the progressive increase of the lipid content of mixed micelles until lipid reorganization in liposomes. Previously, we verified the complete removal of Na cholate occurring by SEC recording FT-IR spectra during the various steps of liposome preparation (De Leo et al., 2014; Mastrogiacomo et al., 2015). Compared to other liposome preparation techniques, the MVT method presents the advantage of producing homogeneous populations of SUVs of controlled size (Holzer et al., 2009) which may improve their mucus-penetrating properties. As shown in Table 1, the size of all liposomes was in a very narrow range (*i.e.*, from 40 nm to 65 nm), and the unmodified ones were characterized by the smaller diameters. Moreover, PDI values ranged between 0.25 and 0.30 for all vesicles investigated suggesting a slightly broad size distribution. A slight electric charge on the vesicle surface was measured even though the zeta potential for all the liposomes was essentially close to zero, as expected for vesicles constituted by PC. Although high zeta potentials (absolute values) are desirable for the physical stability of the colloidal system, liposomes with a high negative surface net charge are easier recognized by macrophages and faster cleared by the blood circulation (Nishikawa et al., 1990; Senior, 1987). The surfactant concentration in PF-LIPOSOMEs was 0.5 mg/mL, a concentration below the critical micelle one (*i.e.*, 0.7% w/v) (Chieng and Chen, 2009). Therefore, it is expected that, as shown in Fig. 1, the hydrophobic region of PF127 is intercalated into the phospholipid palisade of vesicles. PEG-LIPOSOMEs were obtained by adding a PEG-2000-functionalized phosphatidylethanolamine (PE) to the lipid blend. Thus, the hydrophobic tails of PEG-2000 PE lipid are interposed into the lipid bilayer, while its hydrophilic region protrudes into the aqueous compartment (Fig. 1).

Interestingly, a clear dependence of the EE on the drug/lipid ratio was observed. By using a BDP/total lipids ratio up to 10 µg/mg, the EE was of 100, 83 and 80% for PLAIN-, PEG- and PF-LIPOSOMEs respectively, whereas it progressively decreased by enhancing that ratio (Table 2, Table 3 and data not shown). This result could be justified by considering that BDP is a lipophilic molecule and hence it is located within the phospholipid bilayer. Likewise, beyond a 10 µg/mg ratio, the saturation of lipid phase may occur with the consequent separation of the surplus of BDP, which is removed from the liposomes during the SEC step (see Section 2.2). In this regard, however, recent findings of literature about BDP entrapment in liposomes report that BDP tends to form crystals which are adsorbed on vesicle surfaces rather than being entrapped in the bilayers (Subramanian et al., 2016). Nevertheless, we are quite confident that excess BDP possibly not encapsulated at high drug-to-lipid ratio and therefore prone to precipitate as crystals, is effectively removed during liposome preparation. In fact, in the MVT method, liposomes are formed directly in the SEC column during a micelle-vesicle transition induced by detergent separation. The nanometric-sized hydrophobic BDP crystals (Batavia et al., 2001; Khan et al., 2015) possibly expelled from the bilayer during this phase cannot be eluted by the mobile phase together with liposomes since they remain trapped in the meshes of the resin. Moreover, the TEM images did not reveal the presence of solid BDP crystals attached to the vesicles (Fig. 2). These considerations lead us to believe that all the BDP quantified during our experiments is within the bilayer. This hypothesis is also supported by the fact that the percentages in moles of BDP relevant to our preparations with EE equal to 100% (about 1.5% mole) are similar to those that other authors consider below liposomes saturation limit (Batavia et al., 2001; Khan et al., 2015).

Regarding the *in vitro* BDP release study, it should be pointed out that a sustained drug release was noted under the employed experimental conditions for which about 1% of the initially loaded drug was released from PEG- and from PLAIN-LIPOSOMEs and even less (about 0.2% of the initially loaded drug) from PF-LIPOSOMEs. Actually, these small amounts of BDP released are not surprising taking into account that only 1% of organic solvent (ethanol) was used in the release medium. This small amount of EtOH was required in order to dissolve the hydrophobic glucocorticoid BDP, but overall, SLF was almost unmodified in its composition. It is well known, indeed, that the amount of BDP released is directly dependent on the amount of the organic solvent employed (Triolo et al., 2017). On the other hand, it should be also considered that the release conditions are expected to change markedly *in vivo* and BDP release could be triggered in a different way from that predicted by *in vitro* release studies. Indeed, it is well established, that in *in vivo* conditions the cargo release from liposomes could occur by endocytosis of vesicles, a process involving formation of intracellular vesicles as endosomes at first and then lysosomes from which the cargo is released through a pH-dependent mechanism (De Leo et al., 2017b; De Leo et al., 2017c; Sahay et al., 2010).Therefore, from a therapeutic effect point of view, no conclusion should be drawn from the low BDP percentages observed in the *in vitro* release studies which suggest only a sustained release kinetic. In fact, the ability of liposomes to enter the cell with their drug load may increase their release capability compared to that shown by *in vitro* tests.

As mentioned above, the nature of the surface modifier greatly affects the amount of released drug (Fig. 3). Indeed, the drug release from PF-LIPOSOMEs was lower than that from PEG- and from PLAIN-LIPOSOMEs. This lower drug release from PF-LIPOSOMEs may be related to

the increased viscosity of surface modifier PF127 at temperatures close to 37 °C, as below mentioned.

Several methods have been proposed for determining nanocarrier diffusion through the mucus *in vitro* and *in vivo* and they have been recently reviewed by Bernkop-Schnürch and coworkers (Grießinger et al., 2015). Among the *in vitro* methods, the most common approaches exploit the Transwell diffusion systems or modified Ussing chambers (i.e., Transwell-Snapwell system) or Franz diffusion cells (Grießinger et al., 2015; Li et al., 2011). In this paper, we used the Transwell diffusion system to assess the mucus-penetrating properties of liposomes. Very low diffusion of vesicles through the pathological mucus layer was observed up to 21 h, whereas the diffusion efficiency significantly increased between 21 h and 27 h (Fig. 6). The PEG-LIPOSOMEs were the most mucus-penetrating vesicles, with a penetration of 18.8% at 27 h, while the PLAIN-and PF-LIPOSOMEs showed a penetration of 5.3% and 1.2% respectively. The observed penetration kinetics were slower than those observed by others (Bourganis et al., 2015) for PLGA-PEG NPs permeating the porcine intestinal mucus. This outcome could be mainly ascribed to the very thick and viscous mucus used in our experiments, which was overlaid onto the Transwell without diluting it, thus mimicking the real mucus barrier in a patient afflicted by COPD, and highlighting the novelty of our approach. In this regard, it is noteworthy that the mucociliary clearance is severely affected in chronic respiratory diseases (Boucher, 2007; Fahy and Dickey, 2010; Munkholm and Mortensen, 2014). Thus, this altered clearance may allow liposomes to penetrate sputum and land on the airway epithelium also at > 24 h. Our results also indicate that the diffusion of PF-LIPOSOMEs through the pathological mucus was the most restricted. This outcome appeared somewhat surprising, since surface amphiphilic nonionic polymer-modified particles are proved to possess good mucus-penetrating properties. The restricted diffusion of PF-LIPOSOMEs may be partially related to the thermo-sensitive properties of this triblock copolymer. Indeed, as previously described (Tirnaksiz and Robinson, 2005), the viscosity of PF-127 increases at temperatures close to 37 °C and it negatively further affects the diffusion of these particles not only from drug release kinetic point of view (as above mentioned) but also in the mucus permeation process.

Several studies showed that, besides the effect of the particle size (Lai et al., 2007; Lai et al., 2009; Sanders et al., 2000; Suk et al., 2009), an efficient diffusion through the mucus can be achieved by coating particles with PEG of low molecular weight to avoid interactions with mucins (Suk et al., 2009; Yang et al., 2011). In particular, its molecular weight should be not greater than 5 kDa for an optimal mucus diffusion (Bourganis et al., 2015). The conformation of the PEG chains on the particle surface affects the mucus-penetration properties of the particles (Yoncheva et al., 2005). A “brush” conformation of the PEG chains, i.e. densely grafted on the surface of vesicles, facilitates their mucus-penetration capability, while a “mushroom” conformation, i.e. thinly grafted on the surface of vesicles, induces the vesicles adhesion to the mucus components as well as their physical instability (Yoncheva et al., 2005). The PEG chains in PEG-LIPOSOMEs prepared by MVT method should be in brush-like conformation being their molecular weight of 2 kDa but, probably, with PEG chains not highly densely grafted on the surface of vesicles. Therefore, these vesicles, due to their surface features, should penetrate through the highly viscoelastic pathological mucus even though in moderate extent. For the surface unmodified vesicles, a lower mucus-penetrating capability was observed, which was intermediate between those of PEG- and PF-LIPOSOMEs. In this case, indeed, a limited steric obstruction is expected, thanks to their average diameters of about 50 nm.

As above mentioned, liposome-cell interaction and internalization commonly occurs by vesicle adsorption onto cell surface and their subsequent endocytosis (Bozzuto and Molinari, 2015). Several variables affect the endocytic process including size, surface charge, shape and particle composition (Agarwal et al., 2013; Bozzuto and Molinari, 2015). Despite numerous efforts have been carried out to gain information on the role of each variable, in most cases the obtained results are sometimes conflicting and even inconclusive (Bozzuto and Molinari, 2015). The cellular uptake of unmodified vesicles by bronchiolar epithelial H441 cells at physiological temperature (37 °C) was found significantly higher than that observed for the surface modified ones. Moreover, the uptake of PF-LIPOSOMEs was lower than that showed by the PEG-LIPOSOMEs. However, it is well known that PEG meddles in cellular uptake, by interfering with the endocytosis mechanism, as proved for PEG-LIPOSOMEs (Mishra et al., 2004). As to the low uptake of PF-LIPOSOMEs, it has been shown that when PF-127 was associated only with the outer leaflet of the liposomal bilayer it decreased cellular uptake, likely because it diffused from the liposome and stabilized the membrane structure (Li et al., 2011). The decreased cell uptake showed by PEG-LIPOSOMEs compared to the unmodified vesicles may raise the question if the amount of liposomes internalized (22% *vs* 43%) by pulmonary epithelial cells may be useful for a medical application. In this regard, no clear indications exist in the literature, however for gene delivery applications it is accepted that a 10–50% transfection may have appreciable effects from a medical point of view (Shah et al., 2016).

All the liposomes tested showed an excellent *in vitro* biocompatibility towards the bronchiolar epithelial H441 cell line, with a viability > 85% at concentrations ranging between 0.2 and 20 mg/mL BDP as compared to that of the control. These results were confirmed by *in vivo* experiments. Aerosolisation of nanoparticles should be compatible with low toxicity at the respiratory epithelium level. We have previously used the MicroSprayer™ aerosoliser to deliver heparin to the lungs in face of no tissue damage and signs of inflammation (Trapani et al., 2013). We have now extended these observations by assessing the permeability of the endothelial/alveolar barrier through the protein content of BALF, finding that there was no increase in this parameter when liposomes were aerosolised compared to sham controls.

Although we have not specifically studied the issue of lung deposition and persistence of vesicles, others have shown that PEGylated liposomes gives an advantage in that they extend the residence time in the lung and therefore increasing their concentration. It has been reported that nebulization of budesonide encapsulated in PEGylated liposomes has a prolonged therapeutic effect in the lung of experimental animals, with an anti-inflammatory effect equivalent to the standard daily dose of the conventional budesonide formulation (Konduri et al., 2003; Konduri et al., 2005). More recently, PEGylated liposomes showed a strongest tendency to be accumulated to the mouse lungs and retain there at higher concentrations for the longer periods of time compared to mesoporous silica nanoparticles, poly propyleneimine, dendrimer-siRNA complexes nanoparticles, and quantum dots (Garbuzenko et al., 2014).

5. Conclusions

PLAIN-, PEG- and PF-LIPOSOMEs efficiently loaded with BDP (EE very close to 100%) were obtained by MVT, a method that allowed the preparation of SUVs (about 50 nm). The biocompatibility, the drug carrier ability and the stability of these three liposomal formulation were clearly demonstrated. Then, these liposomes were tested as BDP delivery systems able to permeate the pathological pulmonary mucus of hospitalized COPD patients. *In vitro* cytotoxicity studies carried out on H441 cell line by MTT tests showed that all liposomes were not cytotoxic. Intratracheal instillation studies on mice, carried out to assess the plasmatic protein levels in the BALF as a biomarker of injury, showed that both PEG- and PF-LIPOSOMEs did not cause any effect as compared to controls. Moreover, PEG-LIPOSOMES, but not the PF-LIPOSOMEs, show moderate diffusion ability through the pulmonary mucus of COPD patients. Thus, it appears that such lipid nanocarriers moved slower than PEG coated polymeric nanoparticles considering the penetration observed through the mucus from CF patients (Suk et al., 2009). However, taking into account that the

mucociliary clearance is severely reduced in asthma, CF and COPD (Boucher, 2007; Fahy and Dickey, 2010; Munkholm and Mortensen, 2014) and therefore it is reasonable to assume that the consequent vesicle removal should be absent or negligible, it appears that PEG-LIPOSOMEs may possess a good therapeutic potential for the treatment of chronic respiratory diseases.

Taking all the above points into consideration, we can conclude that PEG-LIPOSOMEs have overall the most interesting features among the tested liposomes and more precisely, greater penetration through the pathologic sputum (about 18% of mucus penetration after 27 h *vs* about 5% of PLAIN-LIPOSOMES), appreciable uptake by airway epithelial cells (about 20% of uptake *vs* 40% of PLAIN-LIPOSOMES) and lack of (cyto)-toxicity (essentially no effect on BALF proteins as compared with the controls, while PLAIN-LIPOSOMES determined a little increase in the BALF protein concentration). Therefore, they could represent a new significant perspective in the area of the pulmonary administration. It should be also taken into account, indeed, that previous literature reports about PEG-LIPOSOMES pointed out that they possess a strong tendency to be accumulated in the lungs when administered by inhalation and it may be advantageous for the treatment of lung cancer (Garbuzenko et al., 2014). On the other hand, contrasting results are reported in literature on the use of PEG-LIPOSOMES about their stability during nebulization by commonly used nebulizers (Konduri et al., 2005; Lehofer et al., 2014). However, it can be expected that PEG-LIPOSOMEs could be very promising biomaterials for mucus penetration purposes if prepared with PEG chains in the “brush” conformation, i.e. densely grafted on their surface, besides a particle size of about 50 nm. It suggests the need to improve our method to prepare small vesicles with appropriate surface PEG chain conformations and grafting. Future work should be pointed towards this goal.

Conflict of interest

The authors declare no competing financial interest.

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