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Abstract

Secretory leukocyte protease inhibitor (SLPI) is an endogenous serine protease inhibitor that protects the lungs from excessive tissue damage caused by leukocyte proteases released during inflammation. Recombinant SLPI (rSLPI) has shown potential as a treatment for inflammatory lung conditions. To date, its clinical application has been limited by rapid enzymatic cleavage by cathepsins and rapid clearance from the lungs after inhalation. In this study, rSLPI was encapsulated in 1,2-Dioleoyl-sn-Glycero-3-[Phospho-L-Serine] : Cholesterol (DOPS : Chol) liposomes for inhalation. Incubation of rSLPI with cathepsin L leads to complete loss of activity while encapsulation of rSLPI in DOPS : Chol liposomes retained 92.6% of its activity after challenge with cathepsin L. rSLPI-loaded liposomes were aerosolized efficiently using a standard nebulizer with a minimal loss of activity and stability. This formulation was biocompatible and encapsulation did not appear to diminish access to intracellular sites of action in *in vitro* cell culture studies. Liposome encapsulation of rSLPI therefore improves stability and potentially reduces the level and frequency of dosing required for therapeutic effect after inhalation.

Key words: *rSLPI, liposome, lung, nebulization, aerosol, cathepsin L*

Abbreviations: *Anti-NE, Anti-neutrophil elastase; BAL, Bronchoalveolar lavage; Cat L, Cathepsin L; Chol, Cholesterol; DMEM, Dulbecco's modified eagle's medium; DOPC, 1,2-Dioleoyl-sn-Glycero-3-Phosphocholine; DOPC-rSLPI, rSLPI encapsulated in DOPC : Chol liposomes; DOPS, 1,2-Dioleoyl-sn-Glycero-3-[Phospho-L-Serine]; DOPS-rSLPI, rSLPI encapsulated in DOPS : Chol liposomes; ED, Emitted dose; ELF, Epithelial lining fluid; FCS, Foetal calf serum; LUVs, Large unilamellar vesicles; NE, Neutrophil elastase; NF-κB, Nuclear factor-κB; NRF, Non-respirable fraction; RF, Respirable fraction; rSLPI, Recombinant secretory leukocyte protease inhibitor; SLPI, Secretory leukocyte protease inhibitor; TEM, Transmission Electron Microscopy; TSI, Twin stage impinger.*

Introduction

Local drug delivery to the lungs is an effective means of treating a range of pulmonary diseases such as asthma, cystic fibrosis, bronchitis and emphysema (Suarez and Hickey 2000). Inhalation therapy is typically reserved for delivery of small molecules such as corticosteroids and bronchodilators, however recent developments in aerosol science have made possible the pulmonary administration of proteins and peptides for both systemic and local therapeutic effect. Recombinant human deoxyribonuclease

(rhDNase) was the first protein approved for local inhalation therapy for the treatment of cystic fibrosis and insulin, for the treatment of diabetes, was the first protein approved for marketing for systemic delivery via the lungs. A range of peptides and proteins are currently being investigated for inhalation including recombinant Secretory Leukocyte Protease Inhibitor (rSLPI).

rSLPI is an 11.75 kDa protein found intracellularly in the serous cells of submucosal tracheal and bronchial glands and in non-ciliated cells of the bronchus and bronchial epithelium. Produced by epithelial cells, macrophages

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and neutrophils, it is known to protect the lungs from excessive tissue damage caused by leukocyte proteases during inflammation (Yang et al. 2005). Its abundance in the upper respiratory tract suggests that the primary role of SLPI is to provide an anti-Neutrophil Elastase (anti-NE) shield for the tracheobronchial tree (Fryksmark et al. 1982, Mooren et al. 1983). rSLPI has also been found to possess anti-bacterial, anti-viral and anti-inflammatory activity including the ability to reduce nuclear factor-κB (NF-κB) activation intracellularly (McNeely et al. 1995, Jin et al. 1997, Zhang et al. 1997, Lentsch et al. 1999, Taggart et al. 2002, Greene et al. 2004). rSLPI has therefore been investigated as a potential therapeutic for inflammatory lung disease (McElvaney et al. 1993, Gillissen et al. 1993, Barrios et al. 1998, Wright et al. 1999, Forteza et al. 2001). Current anti-inflammatory treatments such as inhaled corticosteroids and sodium cromoglycate are used to alleviate inflammation in the lungs; however, studies have demonstrated that the use of corticosteroids shows no reduction in disease progression (Adcock 1996, Keatings et al. 1997). Certain physical properties of rSLPI promote its use as a therapeutic agent over other endogenous protease inhibitors, including its stability in acidic pH. This allows rSLPI to remain functionally active in the acidic microenvironment of inflammatory conditions (Swallow et al. 1992, Kottyan et al. 2007). A pI > 9 permits the binding of rSLPI to tissue sites occupied by pro-inflammatory proteases (Vogelmeier et al. 1990). Also the non-glycosylated nature of native SLPI makes the recombinant form identical to its precursor.

Delivery of rSLPI directly to the lungs via inhalation increases targeting to its site of action and has shown an increased half-life over intravenous administration (Bergenfeldt et al. 1990, Stolk et al. 1995). rSLPI has previously been successfully delivered by liquid aerosol *in vivo* with promising therapeutic efficacy (Vogelmeier et al. 1990, 1996, Wright et al. 1999, Forteza et al. 2001). Wright et al. (1999) demonstrated that intratracheal instillation of rSLPI resulted in a pronounced reduction in airway bronchoconstriction and hyper-responsiveness to allergen in an animal asthma model, while McElvaney et al. (1992, 1993) demonstrated its capacity for modulating airway inflammation in patients with cystic fibrosis by reducing neutrophil elastase (NE) activity as well as interleukin-8 levels.

The success of inhaled rSLPI therapy has been limited, however, by rapid clearance and extensive degradation by proteases, particularly cathepsins. Cathepsins are cysteinyl proteases with the exception of cathepsin G which is a serine protease. The cysteinly proteases play a deleterious role in lung inflammation. Cathepsins B, S and L are observed in significantly elevated levels in the epithelial lining fluid (ELF) of the lungs of smokers and cathepsin L (Cat L) levels are known to be increased in the ELF of patients with emphysema (Takahashi et al. 1993, Takeyabu et al. 1998, Taggart et al. 2001). Cathepsin L has been shown to inactivate rSLPI both *in vitro* and *in vivo* (Taggart et al. 2001). This inactivation reduces the NE inhibitory activity available in pulmonary disease states where cathepsins are present in such abundance, thereby perpetuating the protease-to-anti-protease imbalance in the lungs. Treatment of lung inflammation with rSLPI has been demonstrated to be a potential means of alleviating inflammation, however in order to overcome rapid degradation of rSLPI by cathepsins *in vivo*, large doses would be required for inhalation. In this paper the encapsulation of rSLPI within a liposome carrier is assessed as a possible strategy to improve rSLPI stability against Cat L degradation.

Liposomes have advantages over other potential vehicles for lung targeting including sustained release delivery of their cargo in the lungs, increased drug residence time in the lungs (McCullough and Juliano 1979, Morimoto and Adachi 1982, Taylor et al. 1989, Couvreur et al. 1991, Khanna et al. 1996, Saari et al. 1998, Bi and Zhang 2007), improved stability of the drug both *in vitro* and *in vivo*, improved biocompatibility (Niven and Schreier 1990), bioavailability (Huang and Wang 2006), local targeting providing increased potency and reduced toxicity (Wyde et al. 1988, Gruber et al. 1989, Clark et al. 1991, Freise et al. 1994, Khanna et al. 1996, Griffiths et al. 1999, Letsou et al. 1999, Gavalda et al. 2005a, 2005b). Also, the high loading capacity of liposomes and low excipient-to-drug ratio of lipid based carriers results in less excipient accumulation in the lungs after repeated administration compared to polymer-based carriers (Bhavane et al. 2003). These qualities have been observed with liposomal delivery of many therapeutic agents such as amphoteracin B, rifampicin, gentamicin, beclomethasone and therapeutic proteins such as insulin. Their application for the delivery of peptides or proteins to the lungs, however, has yet to be fully explored.

The overall aim of this study was to encapsulate rSLPI in a liposome carrier suitable for inhalation. To this end the goal was to develop a biocompatible liposome system that improved rSLPI stability and enabled high delivery efficiency, without compromising rSLPI's ability to access its site of action extra- and intra-cellularly in alveolar macrophages and lung epithelial cells.

Materials and methods

Materials

RPMI 1640 medium supplemented with L-glutamine 2 m$_M$; Ham's F12, penicillin G, streptomycin sulphate and Dulbecco's Modified Eagle's Medium (DMEM)

(Bio-Science®); U937 cells and Calu-3 cells (American Type Culture Collection®, Manassas, VA); FluoReporter Fluorescein-EX Protein Labelling Kit (F-6433; Molecular Probes®); Spin-out® GT-600 Medi column for purification of proteins > 6000 Mw (Geno-Tech®); 1,2-Dioleoyl-sn-Glycero-3-Phosphocholine (DOPC), Cholesterol and 1,2-Dioleoyl-sn-Glycero-3-[Phospho-L-Serine] (DOPS) (Avanti Polar–Lipids® Inc.); Recombinant human rSLPI was a gift from Amgen® (Thousand Oaks, CA); Human Sputum Leukocyte Elastase (Elastin Products Company®, Missouri); Rabbit anti-SLPI polyclonal IgG and goat anti-rabbit IgG-HRP antibody (Santa Cruz Biotechnology® Inc.); Super-Signal West Pico Chemiluminescent S (Medical Supply Company®, Dublin, Ireland). Cathepsin L, N-(Methoxysuccinyl)-Ala-Ala-Pro-Val 4-nitroanilide and all other reagents were obtained from Sigma-Aldrich® (Tallaght, Dublin, Ireland).

Methods

Liposome preparation. 1,2-Dioleoyl-sn-Glycero-3-Phosphocholine : Cholesterol (DOPC : Chol) and 1,2-Dioleoyl-sn-Glycero-3-[Phospho-L-Serine] : Cholesterol (DOPS : Chol) liposomes were prepared by the conventional thin film hydration procedure as described elsewhere (Amselem et al. 1990). Briefly DOPS or DOPC was mixed with Cholesterol at a ratio of 7 : 3 and dissolved in Chloroform : Methanol (2 : 1). Solvent was removed by evaporation using a rotary evaporator. rSLPI was incorporated into the formulation in a rehydrating buffer (Phosphate Buffered Saline (PBS), pH 7.4). Size reduction of the liposome formulation was achieved by extrusion using a mini-extruder (Avanti Polar-Lipids Inc.). Non-entrapped protein was removed by centrifugation at 45 000 rpm at 4°C for 2 h for DOPC-based liposomes and 40 min for DOPS-based liposomes. The supernatant was removed and the pellet washed with PBS and re-centrifuged. This step was repeated for a further two washes.

Encapsulation efficiency (%EE) of rSLPI in liposomes. The encapsulation efficiency of rSLPI in DOPC : Chol and DOPS : Chol liposomes was determined by reverse phase-HPLC (RP-HPLC). Liposomes were disrupted using 0.5% Triton X and loaded onto a Vydac narrow bore C_{18} column (#218TP5205, Vydac®, Hesperia, CA) for RP-HPLC analysis using a slightly modified procedure previously described (Taggart et al. 2000). Briefly, gradient elution occurred over 40 min using a mobile phase of water and acetonitrile with 0.1% trifluoroacetic acid. Area under the curve was analysed at 214 nm. The supernatant samples were analysed by RP-HPLC;%EE was defined as the rSLPI encapsulated in liposomes as a percentage of loading dose.

Phospholipid assay. The Stewart (1980) assay was used to determine the concentration of phospholipid present in the liposomal formulations. Briefly, 2 ml chloroform and 2 ml ferrothiocyanate reagent and 0.1 ml of liposome sample were vortexed vigorously for 1 min. The resultant mixture was centrifuged at 1000 rpm for 5 min and the lower chloroform layer removed by glass pipette and measured at 485 nm. Based on a standard curve for the appropriate lipid, the concentration of phospholipid present in the sample was calculated.

Size distribution and surface charge analysis. Size analysis and ζ-potential of the liposomes were determined by dynamic light scattering (DLS) (HPPS®, Malvern Instruments) and laser Doppler electrophoresis (LDE) (Zetasizer Nano ZS®, Malvern Instruments), respectively. DLS measures the fluctuation in intensity of scattered light caused by particle movement. This method can measure particle size ranges of 0.6–6000 nm (Malvern Instruments Ltd. 2003) and therefore is a very quick and straightforward means of characterizing nanoparticles (Mehnert and Mader 2001). Transmission electron microscopy (TEM) was used to confirm morphology and size. Samples were placed onto a 400-mesh butvar-coated copper grid for 2 min then washed with deionized water. The grid was negatively stained with a drop of uranyl acetate (0.5%w/v) for 2 min and blotted using a clean paper towel. The liposomes were viewed using a Hitachi H7000 Transmission Electron Microscope.

Western blot analysis. Stability of rSLPI was determined by western blot analysis. Samples and standards containing 125 ng rSLPI were electrophoresed on 15% polyacrylamide gel and blotted onto nitrocellulose. After blocking in I-block®, rSLPI was detected using affinity purified rabbit anti-SLPI polyclonal IgG (1 : 1000 in I-block) for 1 h followed by incubation with goat anti-rabbit IgG-HRP antibody (1 : 7500) for 1 h. Development was carried out using SuperSignal West Pico® chemiluminescent substrate kit (Taggart et al. 2001).

Anti-neutrophil elastase (Anti-NE) activity assay. Activity of rSLPI was assayed by measuring its inhibition of human neutrophil elastase (NE) activity on the substrate N-methoxy-succinyl-Pro-Ala-Ala-Val-p-nitroanilide. rSLPI was incubated with NE at room temperature for 5 min. Upon addition of the substrate, the change in absorbance (ΔAbs) at 405 nm was measured from T_0 to T_{5min} (McElvaney et al. 1992).

Cathepsin L inactivation of rSLPI. Inactivation of rSLPI by cathepsin L was determined by measuring NE inhibitory activity following incubation of 500 ng rSLPI with 50 ng cathepsin L as previously described

(Taggart et al. 2001). Incubation occurred for 2 h at 37°C in 0.1 M sodium acetate buffer, pH 5.5, containing 1 mM EDTA, 10 mM dithiothreitol and prepared to a final volume of 250 μl. The reaction was stopped by addition of 0.2 M Tris, pH8.5 containing 1 μM E-64. The samples and controls were then assayed for anti-NE activity.

Cell viability studies.   Two cell lines, an airway epithelial cell model, Calu-3 cells, and a monocyte model, U937 cells, were used to assess cell viability after incubation with DOPS-rSLPI. Calu-3 airway epithelial cell established by Fogh et al. (1977), were grown in 150 cm² flasks and maintained in a 1 : 1 mixture of Ham's F12 : DMEM containing 10% foetal bovine serum (FBS), 100 μg ml⁻¹ penicillin G and 100 μg ml⁻¹ streptomycin sulphate. Calu-3 cell viability was tested using the Thiazolyl Blue (MTT) assay (Mosmann 1983). Briefly, cells were seeded in 96 well tissue culture plates and grown to 90% confluency. The cell medium was removed and replaced with fresh medium containing DOPS-rSLPI (0.64–10.25 μg ml⁻¹ rSLPI and 10–400 μM lipid). After an 18 h incubation period the media was removed and the cells were washed with PBS; 100 μl of a 1 mg ml⁻¹ MTT reagent was added to each well and left overnight; 100 μl SDS 10% w/v in 0.1 M HCl was added to lyse the cells. The wells were read at 572 nm. U937 monocytes established by Sundstrom and Nilsson (1976) were cultured in RPMI-1640 media containing 2 mM glutamine supplemented with 10% FCS, 100 μg ml⁻¹ penicillin G and 100 μg ml⁻¹ streptomycin sulphate. The monocytes were incubated with equivalent concentrations of DOPS-rSLPI to that for Calu-3 cells and incubated at 37°C for 18 h. Equal volumes of cell and Trypan blue were mixed. Both dead and live cells were counted using a haemocytometer.

Jet nebulization of rSLPI encapsulated in DOPS : Chol liposomes.   Jet nebulization, via the Acorn II® nebulizer (Model No. 124015, Marquest Medical Products Inc, CO), was used to aerosolize the DOPS : Chol rSLPI liposomes using an air pressure of 40 psi; 5 ml of DOPS-rSLPI liposomes was placed into the nebulizer device and attached to a twin stage impinger (TSI; European Pharmacopoeia Apparatus A) using a flow rate of 60 L min⁻¹. The upper and lower chambers of the impinger were assayed for rSLPI content after disrupting the liposomes with 0.5% Triton X. Emitted dose and respirable (RF) and non-respirable (NRF) as a percentage of emitted dose were calculated. Stability analysis of rSLPI post-nebulization was carried out by western blot, RP-HPLC and anti-NE activity assay. A lipid assay was conducted to assess the co-distribution of lipid with rSLPI after nebulization. A sample was removed from the TSI chambers, centrifuged and the supernatant assayed to assess whether any leakage of rSLPI from its liposomal carrier had occurred during nebulization.

Uptake into Calu-3 airway epithelial cells and U937 monocytes.   rSLPI was fluorescently labelled with a dye reagent Fluorescein-EX (Invitrogen). The reactive succinimidyl ester moiety of this dye reagent reacts with the primary amines of the protein to form a stable rSLPI-fluorescein conjugate. Free dye was removed using a Spin-OUT column (Geno Technology) with a molecular weight cut-off of 6000. The yield after purification was quantified using a protein assay (Bio-Rad laboratories).

rSLPI uptake into U937 monocytes was determined by incubating 10⁶ ml⁻¹ cells with 10 μg ml⁻¹ rSLPI-fluorescein (encapsulated or free) for 2 h. The cells were washed three times in PBS; 10 000 cells were then spun onto glass microscope slides and air-dried. The cells were incubated for 5 min in 1% w/v paraformaldehyde followed by rinsing three times in PBS. Finally, some slides were incubated with 0.5 μM propidium iodide for 20 min. Fluorescence-free glycerol-based mounting medium was added before covering the slide with a coverslip for visualization. The cells were viewed using a fluorescence microscope (Axiovert 200; Carl Zeiss MicroImaging Inc., Germany).

Calu-3 airway epithelial cells were seeded at a density of 104 cells/well onto coverslips in wells of a 6 well plate and then incubated with 10 μg/ml rSLPI-fluorescein (encapsulated or free) for 2 h, followed by processing and visualization as outlined above.

Statistical analysis of results.   Results were expressed as mean ± standard deviation. Where appropriate the unpaired *t*-test was used to determine the significance of results. In all cases, a probability value of less than 0.05 was considered to be significant.

Results

Optimization of liposome encapsulation of rSLPI

Encapsulation of rSLPI in 1,2-Dioleoyl-sn-Glycero-3-Phosphocholine : Cholesterol liposomes, which will hereon be referred to as DOPC-rSLPI, or 1,2-Dioleoyl-sn-Glycero-3-[Phospho-L-Serine] : Cholesterol liposomes, which will hereon be referred to as DOPS-rSLPI, was optimized. Extrusion of the liposomal formulations resulted in large unilamellar vesicles (LUVs) with an average diameter of less than 200 nm, as determined by dynamic light scattering (Table 1) and TEM.

Encapsulation efficiency (%EE) was used to express rSLPI encapsulation into liposomes as a percentage of rSLPI added to the formulation. %EE of rSLPI in the two liposome systems varied significantly ($p < 0.0001$)

depending on the lipoidal composition of the carrier (Table 1). %EE of rSLPI in DOPS : Chol liposomes was $74.1 \pm 2.97\%$ compared to $10.3 \pm 0.34\%$ for DOPC : Chol liposomes.

Stability and activity of rSLPI after encapsulation

The stability of rSLPI after encapsulation was confirmed by RP-HPLC and western blot analysis (Figure 1). The retention of anti-protease activity was confirmed by assessing anti-NE activity of the encapsulated rSLPI in PBS Figure 2.

Stability against cathepsin L degradation

To assess the protective effect of the liposomes, free and encapsulated rSLPI was incubated with cathepsin L (Cat L) for 2 h at 37°C. Incubation of unencapsulated rSLPI with Cat L causes its activity to be destroyed (0% activity

retained), whereas rSLPI encapsulation resulted in retention of anti-NE activity after incubation with Cat L (Figure 2). The protective capacity of DOPS-rSLPI was significantly greater at $92.6 \pm 4.4\%$ rSLPI activity retained compared to that observed for DOPC-rSLPI ($49.6 \pm 4.3\%$; $p < 0.0001$) (Figure 2).

Stability and activity of DOPS-rSLPI during storage

The stability of the DOPS-rSLPI liposomes after storage for 8 weeks at 4°C was assessed. Although the particle size of the liposomes increased significantly during storage from 153.6 ± 2.5 to 208.91 ± 2.31 nm ($p < 0.0001$), both RP-HPLC and western blot analysis confirmed that rSLPI remained stable under these storage conditions. In addition the anti-NE activity of rSLPI was retained during the storage period. The ability of the DOPS : Chol liposome to protect rSLPI from Cat L degradation, as assessed by rSLPI anti-NE activity assay, diminished to $80.4 \pm 3.5\%$ by week 8, compared to its initial anti-NE activity at time of formulation of $92.6 \pm 10.1\%$ (Figure 3). This diminished protection was due to leakage of rSLPI from the liposomes.

Toxicity studies

No significant cell toxicity was seen after DOPS-rSLPI incubation at concentrations of rSLPI ranging from

Table 1. Encapsulation efficiency (%EE), particle size and ζ-potential of rSLPI in DOPS : Chol or DOPC : Chol liposomes ($n = 3 \pm$ SD).

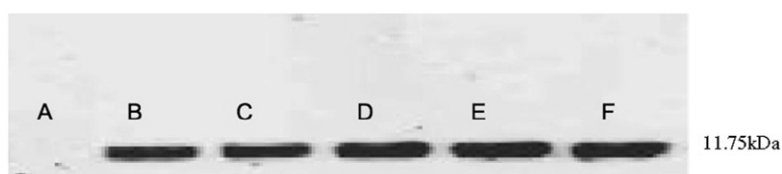| Liposome system	%EE	Size (nm)	ζ-potential (mV)
DOPC : Chol + rSLPI	$10.3 \pm 0.34\%$	184.1 ± 0.55	-8.67 ± 0.654
DOPS : Chol + rSLPI	$74.1 \pm 2.97\%$	153.6 ± 2.47	-58.80 ± 1.458

Figure 1. Western blot analysis of blank DOPS : Chol liposomes (A); rSLPI encapsulated in DOPS : Chol liposomes (B–D); and unencapsulated rSLPI (E, F).
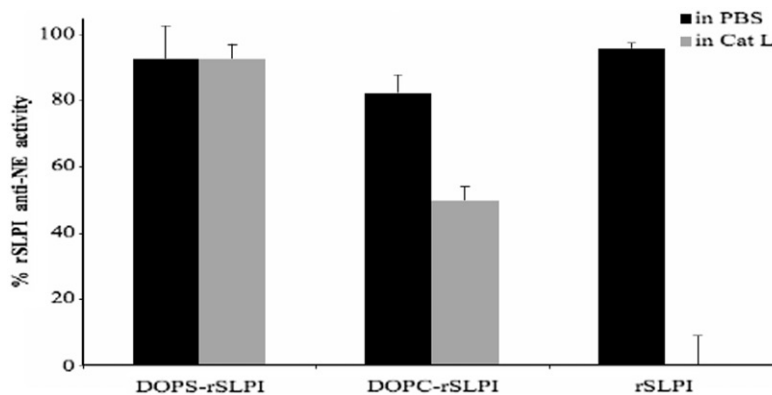

Figure 2. Anti-NE activity of 200 ng rSLPI or equivalent encapsulated rSLPI after incubation with or without cathepsin L for 2 h at 37°C ($n = 5 \pm$ SD).
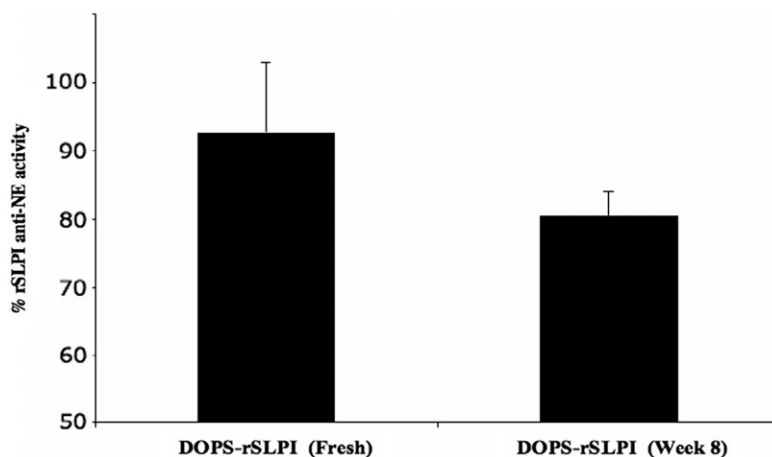
Figure 3. Anti-NE activity of freshly prepared DOPS-rSLPI (Fresh) or DOPS-rSLPI stored at 4°C for 8 weeks (week 8) after incubation with Cat L $(n = 3 \pm SD)$..

0.65–10.25 µg ml^{-1} for 18 h at 37°C in either an epithelial or monocyte cell model.

Nebulization into a twin stage impinger (TSI) using a jet nebulizer

Jet nebulization of rSLPI in DOPS:Chol liposomes resulted in an emitted dose (ED) of rSLPI of 49.5 ± 4.9%, the majority of which (90.6 ± 1.6%) reached the lower stage of the TSI, this constituted the respirable fraction (RF), the remainder (9.4 ± 1.6%) depositing in the upper stage, the non-respirable fraction (NRF).

In order to determine if leakage occurred, lipid content was also assayed. ED of the liposome carrier was 85 ± 2.9%. Liposome deposition in the RF was 83.7 ± 4.9% with 16.3 ± 4.9% in the NRF stage of the TSI. This confirmed concurrent liposome and rSLPI deposition in respirable and non-respirable fractions. The greater emission of lipid from the nebuliser device than rSLPI indicated a degree of leakage.

Stability and activity of rSLPI after nebulization

Stability of rSLPI was maintained after nebulization as assessed by RP-HPLC and western blot analysis (Figure 4). The anti-NE activity of rSLPI was also preserved after nebulization. The capacity of the DOPS liposomes to protect rSLPI against Cat L degradation diminished however after nebulization to 46.8 ± 4.8% (Figure 5). Centrifugation of the samples from TSI chambers and RP-HPLC analysis of the supernatant confirmed that 41.1 ± 3.8% of rSLPI had leaked from the liposome carrier.

Cellular uptake

Uptake of unencapsulated rSLPI labelled with a fluorescent marker into U937 monocytes and Calu-3 airway epithelial cells was observed after incubation for 2 h at 37°C. Intracellular uptake did not appear to be reduced when rSLPI was encapsulated in the DOPS:Chol liposomal carrier compared with unencapsulated rSLPI (Figure 6).

Discussion

The encapsulation of rSLPI within a carrier to provide an aerosol formulation capable of protecting against Cat L degradation was investigated. Liposomes have previously shown potential at enhancing stability, improving potency, reducing toxicity and providing sustained release in the lungs. Therefore liposomes were selected as the carrier of choice for enhancing the therapeutic potential of rSLPI. SLPI is an endogenous molecule which has already demonstrated application in relieving inflammation in the lungs, however it is prone to rapid enzymatic degradation and clearance in vivo (Taggart et al. 2001).

Large unilamellar vesicles (LUV's) were produced using two types of liposomal formulation, one incorporating DOPS and the second DOPC. Both liposomes contained cholesterol to enhance lipid bilayer strength and liposome integrity (Niven and Schreier 1990, McMullen et al. 1993, Bhattacharya and Haldar 1996). Manufacture of LUV's provided a balance between loading efficiency of liposomes, which increases with increasing size, and liposome stability for which the optimal size is in the range of 80–200 nm (Lasic 1998).
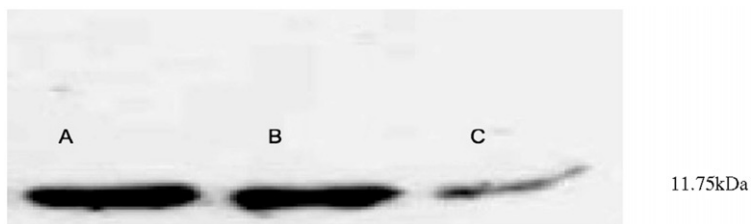
Figure 4. Western blot analysis of DOPS-rSLPI after nebulization liposomes (A, B); and unencapsulated rSLPI (control) (C).
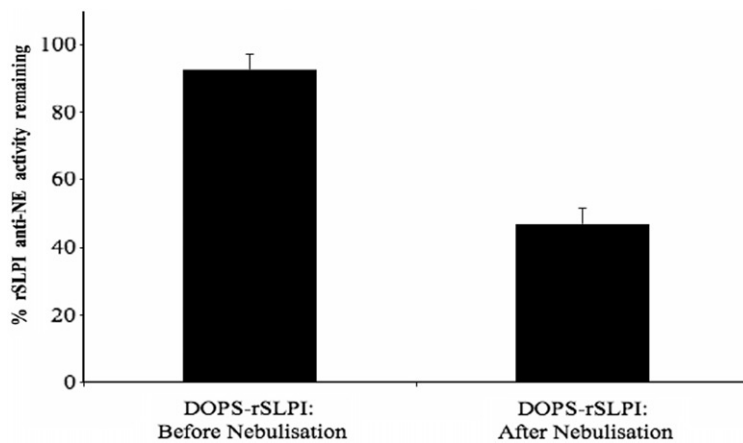

Figure 5. Anti-NE activity remaining after incubation of DOPS-rSLPI samples, before and after jet nebulization, with Cat L for 2 h at 37°C ($n = 3 \pm$ SD).
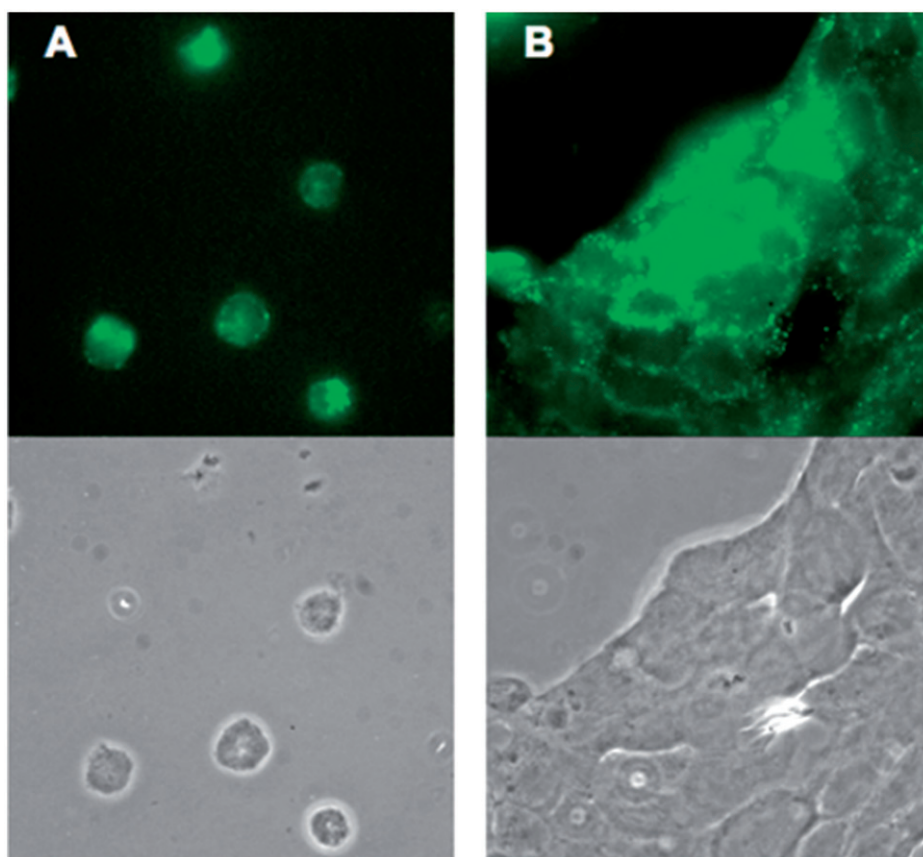

Figure 6. Uptake of rSLPI-fluorescein encapsulated in DOPS:Chol liposomes into (A) U937 monocytes and (B) Calu-3 cells.

The results demonstrate the superiority of the DOPS : Chol liposomes in terms of encapsulation efficiency, size and stability. The difference in %EE was due to ionic interactions between positively charged rSLPI and the negatively charged DOPS lipid. In the case of DOPS, the serine group confers an anionic charge onto the liposome resulting in high %EE of rSLPI within this carrier (%EE of rSLPI in DOPS : Chol liposomes: $74.1 \pm 2.97\%$.). Conversely the DOPC lipid, which is zwitterionic, does not produce significant ionic interactions between the liposome and rSLPI resulting in a relatively low %EE of $10.3 \pm 0.34\%$ (Table 1). Previous studies have demonstrated enhanced encapsulation between cationic therapeutics and anionic liposomes (Lutwyche et al. 1998). The high ζ-potential associated with DOPS-rSLPI (-58.8 mV) and its smaller particle size (Table 1) indicates that this is a more condensed liposome formulation than the DOPC-rSLPI formulation.

After successful encapsulation into both liposome formulations, the stability and activity of rSLPI was confirmed to be comparable to rSLPI pre-encapsulation. The two liposomal systems, however, varied in their ability to protect rSLPI from enzymatic degradation by Cat L when tested *in vitro*. After incubation with Cat L for 2 h at 37°C, the NE inhibitory activity of DOPS-rSLPI was retained at 92.6% compared to 49.6% for DOPC-rSLPI and a complete loss of activity for rSLPI alone (Figure 2). The superior encapsulation capacity and overall rSLPI interaction of the DOPS : Chol liposomes appears, therefore, to offer the greater protection against cathepsin degradation of the liposomes tested.

To assess whether the liposome formulation could retain its stability and activity under typical storage conditions, the formulation was stored at 4°C and analysed weekly. Stability and activity remained constant over an 8-week period, however the particle size the liposomes had increased by a factor of 1.36 by the end of the study. Increases in liposome particle size over time have been observed due to agglomeration and fusion of liposomes during storage (Fan et al. 2007). However, in other studies liposomes remained stable in terms of particle size for at least 5 months when stored at 4°C (Yohannes et al. 2006, Garg et al. 2007). Liposome composition appears to be a key factor, with the inclusion of negatively charged phospholipids, such as DOPS, thought to reduce the tendency for aggregation and particle size increases and reduce the rate of drug leakage during storage (Manosroi et al. 2002). However, in this study the presence of DOPS in the liposome formulation didn't appear to prevent size increases completely and this size increase and instability was corroborated in the drug (rSLPI) leakage evidenced by the decreased protective ability of the liposomes after 8 weeks storage (Figure 3).

The aerodynamic properties of DOPS-rSLPI were assessed using a jet nebulizer to aerosolize the DOPS-rSLPI liposomes. Emitted dose and respirable fraction of rSLPI were acceptable for aerosolization using this device at 49.5% and 90.6%, respectively. A concern related to this nebulization technique is that 99% of the formulation is ultimately refluxed, which can cause denaturation of the protein due to repeated stress and exposure to air–water interfaces (Niven 1995). Denaturation did not occur, however, during rSLPI nebulization and stability and activity were successfully maintained. Disruption to the liposomal bilayer resulting in leakage of encapsulated drug is also a recognized problem relating to jet nebulization (Taylor et al. 1990, Niven et al. 1991). Maintenance of rSLPI encapsulation within the liposome vesicles after nebulization was tested. Overall emitted dose for the liposome carrier was higher than that observed for rSLPI at $85 \pm 2.9\%$, which suggested leakage of the protein from the liposome carrier. Centrifugation of the aerosolized preparations revealed a significant amount of rSLPI leakage, $\sim 40\%$, after nebulization. The percentage of leaked rSLPI correlated directly with the observed reduction in protection of rSLPI by the liposome carrier from Cat L degradation (Figure 5) post-nebulization. A previous study demonstrated nebulization-induced leakage of drugs from liposomal membranes that typically occurs when low concentrations of cholesterol are incorporated into the lipid membrane (Bridges and Taylor 1998). In the case of the DOPS : Chol liposomes formulated in this study, however, cholesterol is used at 30 mol%, a concentration demonstrated to improve the stability of the liposome membrane during nebulization (Bridges and Taylor 1998). The newer generation nebulizers may be the key to reducing leakage from liposome formulations. Studies using electronic nebulization have demonstrated reduced vesicle disruption of liposome formulations while also improving the rate of aerosol output. However, depending on the formulation, the observed emitted dose may be lower, and the aerodynamic diameter of the aerosol droplet produced can be larger than that obtained using jet nebulization (Wagner et al. 2006, Elhissi et al. 2007).

Given that rSLPI acts both intracellularly and extracellularly, the intracellular uptake of rSLPI into airway epithelial cells and monocytes was also evaluated. It appeared that there was no reduction in intracellular uptake of rSLPI after encapsulation in DOPS : Chol liposomes compared to that for unencapsulated protein. Moreover, studies carried out by De Haan et al. (1996) have demonstrated enhanced alveolar macrophage uptake of negatively charged liposomes deposited in the lungs of mice.

Overall, DOPS-rSLPI liposomes provide a highly efficient means of encapsulating rSLPI and thereby protecting it from Cat L degradation. This biocompatible

delivery system is easily aerosolized and is a promising strategy to reduce the dose of rSLPI previously required for inhalation, while also offering the possibility of providing a sustained release formulation for local lung delivery.

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