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Research Paper

Chitosan-coated antifungal formulations for nebulisation

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Abstract

Objectives The aim of this study was to produce and characterise amphotericin B (AmB) containing chitosan-coated liposomes, and to determine their delivery from an air-jet nebuliser.

Methods Soya phosphatidylcholine : AmB (100 : 1) multilamellar vesicles were generated by dispersing ethanol-based proliposomes with 0.9% sodium chloride or different concentrations of chitosan chloride. These liposomes were compared with vesicles produced by the film hydration method and micelles. AmB loading, particle size, zeta potential and antifungal activity were determined for formulations, which were delivered into a two-stage impinger using a jet nebuliser.

Key findings AmB incorporation was highest for liposomes produced from proliposomes and was greatest (approximately 80% loading) in chitosan-coated formulations. Following nebulisation, approximately 60% of the AmB was deposited in the lower stage of the two-stage impinger for liposomal formulations, for which the mean liposome size was reduced. Although AmB loading in deoxycholate micellar formulations was high (99%), a smaller dose of AmB was delivered to the lower stage of the two-stage impinger compared to chitosan-coated liposomes generated from proliposomes. Chitosan-coated and uncoated liposomes loaded with AmB had antifungal activities against *Candida albicans* and *C. tropicalis* similar to AmB deoxycholate micelles, with a minimum inhibitory concentration of 0.5 μg/ml.

Conclusions This study has demonstrated that chitosan-coated liposomes, prepared by an ethanol-based proliposome method, are a promising carrier system for the delivery of AmB using an air-jet nebuliser, having a high drug-loading that is likely to be effectively delivered to the peripheral airways for the treatment of pulmonary fungal infections.

Keywords amphotericin B; antifungal; chitosan; liposome; nebulisers; proliposome

Introduction

Pulmonary delivery of liposome-entrapped drugs may offer therapeutic advantages over inhalation of drug in the free form. Liposomal aerosols with size less than 3–5 μm can be deposited in the deep lung[1] and may localise the action of entrapped materials within the alveolar region for prolonged periods.[2] Liposomes are potentially very suitable carrier systems for inhalation since they can be prepared from phospholipids of the same or similar composition to endogenous lung surfactant, and hence are biocompatible and biodegradable. The pulmonary deposition and clearance of liposomes delivered using air-jet nebulisers are dependent on the liposomal aerosol droplet size,[1] whilst their stability during nebulisation is a function of the air pressure used to produce the aerosols, liposome size and bilayer composition.[3,4]

To overcome the instability of conventional liposomes, such as hydrolysis, oxidation, aggregation and fusion, proliposome technologies have been developed.[5,6] Proliposomes are phospholipid(s) in particulate or solution form that readily generate liposomes on dispersion in aqueous medium. Liposomes produced from ethanol-based proliposomes have been reported to entrap more than 60% salbutamol sulfate and they are efficiently delivered from an air-jet nebuliser.[7]

Invasive fungal infections produce high morbidity and mortality rates, particularly in immunocompromised patients.[8] Currently, systemically administered antifungal therapeutic agents are the main treatment option and these are associated with severe adverse effects. Amphotericin B (AmB), a polyene macrolide antifungal drug, is a first-line therapy for treatment of fungal infections such as candidiasis and pulmonary aspergillosis.

The adverse effects associated with AmB use are many and include nephrotoxicity, which is dose-limiting and may cause permanent harm. The severity of adverse effects, including nephrotoxicity, can be reduced through appropriate formulation, particularly with the use of lipid-based systems.[9,10] There are four commercial AmB formulations: Fungizone (a colloidal dispersion of AmB with sodium deoxycholate), Ambisome (a liposomal AmB formulation), Amphocil and Abelcet, all for systemic administration.

Formulations that localise antifungal drugs within the lungs may minimise adverse effects, and aerosolised liposome AmB formulations have achieved promising results in animal models of pulmonary fungal disease.[11,12] Nebulisation of liposomal formulations containing AmB has been shown to enhance the retention of AmB in the lungs, with no effect on respiratory function[13] and improved prophylaxis against *Aspergillus* spp. compared to AmB deoxycholate.[12] However, whilst nebulisation of both liposomal AmB and non-liposomal AmB deoxycholate delivers high local concentrations, the function of pulmonary surfactant is adversely affected by nebulised AmB deoxycholate. In contrast, liposomal AmB does not disturb surfactant activity.[14,15]

Chitosan was included in the formulations used in the present study because it is a biocompatible, biodegradable and bioadhesive polysaccharide cationic polymer, which may, as in-vitro studies have shown, prolong the contact time of the formulations with mucosal tissues without causing toxic effects or irreversible morphological alterations to pulmonary cells.[16] Previously, inclusion or coating of chitosan in a liposomal formulation for nebulisation improved the mucoadhesiveness of the vesicles and decreased their toxicity to pulmonary epithelial cells.[17] This study aimed to manufacture and characterise chitosan-coated liposomes prepared by an ethanol-based proliposome method, determine their delivery from a jet nebuliser and hence assess their suitability as drug carriers for the pulmonary delivery of AmB by nebulisation.

Materials and Methods

Drugs and reagents

Absolute ethanol and methanol, AnalaR grade, were purchased from BDH, UK. Soya phosphatidylcholine (SPC; Lipoid S-100) was a gift from Lipoid, Switzerland. Dimethylsulfoxide (DMSO) and AmB from *Streptomyces* spp. were purchased from Sigma-Aldrich, UK. AmB-deoxycholate complex was purchased from E.R. Squibb & Sons, UK. Water for injections and 5% glucose injection were purchased from Fisher Scientific, UK. Water-soluble chitosan chloride (Protasan UP Cl 113, molecular weight 50 000 to 150 000 g/mol and apparent viscosity <20 mPa·s) was purchased from Novamatrix Biopolymer, Norway. Pari LC Sprint air-jet nebulisers and Pari TurboBoy N compressor were purchased from Pari, GmbH, Germany.

Amphotericin B deoxycholate micelles

The AmB-deoxycholate complex was reconstituted by addition of sterile water for injections to produce micelles. This was further diluted to the desired concentration, with sterile water for injections for determination of drug loading and aerosolisation properties (0.07 mg/ml), and with 5% glucose injection for antifungal susceptibility testing.

Thin-film hydration liposomes

Soya phosphatidylcholine (SPC, 140 mg) was weighed into a round-bottomed flask and dissolved with ethanol, and then AmB (2 mg in 2 ml dimethylsulfoxide (DMSO)) was added to the ethanolic lipid solution. The flask was attached to a rotary evaporator (Buchi Rotavapor R-114, Buchi, Switzerland) and a vacuum (Buchi Vac V-500, Buchi) was applied. The rotation speed was set at maximum and the water bath temperature was set at 45°C. After 15–20 min, the vacuum was switched off and the flask detached and flushed with nitrogen for 1 min to remove residual solvent. Then 0.9% w/v sodium chloride or 0.2% w/v chitosan chloride solution was added to hydrate the thin film, giving a phospholipid concentration of 14 mg/ml and AmB concentration of 0.143 mg/ml. The flask was vigorously shaken for 10 min, and immersed in a water bath (45°C) for 15 min followed by another 10 min of shaking. The resultant multilamellar vesicles (MLVs) were allowed to anneal for 45 min at 45°C, then probe sonicated before analysis.

Ethanol-based proliposomes

SPC (100 mg) was dissolved in warm ethanol (100 μl). A stock solution of AmB in DMSO with a concentration of 20 mg/ml was prepared, and from that 50 μl was added to the ethanolic SPC solution to give a 1 : 100 ratio (AmB : phospholipid) and mixed using a Rotamixer at maximum setting for 1 min. This yields the proliposome formulation, which can be stored for prolonged periods. The addition of 0.9% w/v sodium chloride or chitosan chloride (0.1, 0.2 or 0.3% w/v) solutions to the proliposomes, followed by mixing using the Rotamixer at maximum setting for 2 min, resulted in the formation of liposomes (total volume 14 ml) with a phospholipid concentration of 7.1 mg/ml and AmB concentration of 0.07 mg/ml. The liposomes were allowed to anneal for 45 min at 45°C, then probe sonicated before analysis.

Sonication of liposomes

A bench-mounted probe sonicator (MSE Soniprep 150, MSE Limited, UK) was used to reduce liposome size. Liposomes were placed in a glass vial surrounded with ice to minimise the temperature effects produced by the titanium probe during sonication. The sonication was at 20 kHz, with wave amplitude of 8 μm and duration of 5 min for each sample. Samples were filtered through 0.22 μm syringe filter units (Millex GP, Millipore, Carrigtwohill, Cork, Ireland) to remove insoluble AmB that was not incorporated into the liposomal bilayers.

Transmission electron microscopy (TEM)

One drop of liposome dispersion was placed on carbon-coated copper grids (400 mesh) (TAAB Laboratories Equipment Ltd, UK), stained with 1% phosphotungstic acid and viewed using a Philips CM 120 Bio-Twin TEM (Philips Electron Optics BV, The Netherlands).

Size analysis of liposomes

The volume median diameter (VMD) and span (90% undersize minus 10% undersize)/VMD) of the unsonicated liposomes were measured by laser diffraction (Malvern Mastersizer S, Malvern Instruments Ltd, UK) using an MS7-magnetically stirred dispersion cell unit (Malvern Instruments Ltd). The size distribution of sonicated and filtered liposomes was obtained as Z_{Ave} hydrodynamic diameter and polydispersity index (PI), using a Malvern 3000 spectrometer (Zetasizer, Malvern Instruments Ltd), which was also used to measure the zeta potential of liposomes.

Amphotericin B loading

The AmB concentration in the liposomal sample was detected using a UV-visible spectrophotometer (Varian Australia Pty Ltd, Australia). The absorbance values of AmB and in the micellar and liposomal formulations were determined in DMSO : methanol (2 : 3) at 410 nm. A calibration curve of AmB concentration against absorbance was constructed, and AmB in the formulations determined with reference to the calibration curve. The drug loading was calculated by comparing the actual AmB concentration to the theoretical concentration of AmB used in formulations.

Aerosol delivery of amphotericin B formulations

The two-stage glass impinger (TSI, Copley Instruments, UK) comprises two stages that 'represent' the upper and lower respiratory tract.[18] The cut-off aerodynamic diameter between stages is 6.4 μm at 60 l/min flow rate. Quantities of 30 and 7 ml of 0.9% w/v sodium chloride or methanol : DMSO solution were placed in the lower and the upper stages, respectively, to collect the nebulised aerosols. After assembling the two stages, 5 ml of micelles (containing 355.0 μg AmB deoxycholate), MLVs (containing 50 mg phospholipid and 336.7 and 396.0 μg AmB dispersed in 0.9% NaCl and 0.2% chitosan chloride, respectively) or liposomes produced from proliposomes (containing 50 mg phospholipid and 333.5, 364.0, 391.6 and 397.5 μg AmB for liposomes prepared in 0.9% NaCl, 0.1, 0.2 and 0.3% chitosan chloride, respectively) was placed in a Pari LC Sprint nebuliser (Pari Gmbh, Germany). This was attached to a TurboBoy N compressor (Pari Gmbh) and the generated aerosol was directed towards the throat of the TSI. Samples for analysis, from the upper and lower stages, were collected after the nebuliser reached dryness. In all cases the total mass balance using the TSI was within the European Pharmacopoeial limit of 75–125% of average delivered dose. Total aerosol mass output was calculated by weighing the nebuliser before and after nebulisation of the formulations.

Aerosol size analysis by laser diffraction

The size distribution of aerosol droplets was analysed using a Malvern 2600C laser diffraction size analyser (Malvern Instruments Ltd) with a 63 mm lens. Preparations (5 ml) were placed into a Pari LC Sprint nebuliser attached to the TurboBoy N compressor. The nebuliser was clamped 2.5 cm from the laser beam and aerosols traversed the beam 2.5 cm from the lens of the instrument. A vacuum was applied to draw aerosols through the beam. The VMD and span were recorded at time intervals until dryness was reached.

In-vitro antifungal susceptibility testing

The antifungal activity of the formulations was assessed against *Candida albicans* ATCC 14053 and *Candida tropicalis* ACTC 730 by microdilution susceptibility testing in 96-well microtitre plates. Tests were performed in a total volume of 200 μl Antibiotic Medium No. 3 (Oxoid, UK), supplemented with 2% glucose and buffered to pH 7 with 10 mM phosphate as described previously.[19] Two-fold serial dilutions of the formulations were evaluated (0.03–16 μg of AmB per millilitre) against a yeast inoculum size of approximately 5×10^3 colony forming units/ml. Plates were incubated at 35°C and read after 24 and 48 h. The minimum inhibitory concentration (MIC) was defined as the lowest concentration of the drug to inhibit visible growth after 48 h.

Statistical analysis

SPSS Version 17.0 (SPSS Inc.) software was used for non-parametrical statistical analysis. The Mann–Whitney statistical test was applied to compare results between two groups. The Kruskal–Wallis followed by post-hoc Nemenyi's statistical tests were used to compare results between different groups. All experiments were undertaken in triplicate.

Results

Amphotericin B deoxycholate micelles

The loading efficiency of AmB into micelles was 99.4 ± 1.4% and the mass output from the nebuliser was 93.7 ± 1.2%. The mean (±SD) VMD and span of the aerosol produced on nebulising the AmB micelles were 3.86 ± 1.8 μm and 2.1 ± 0.09, respectively. After nebulisation to 'dryness', the majority (58%; equivalent to 205.7 ± 7.0 μg) of the delivered AmB was deposited in the lower stage of the TSI, with 27% remaining in the nebuliser chamber (Figure 1).

Amphotericin B liposomes prepared by the thin-film method

Lipososomes produced by the thin-film method incorporated between 47 and 55% AmB (Table 1), with significantly

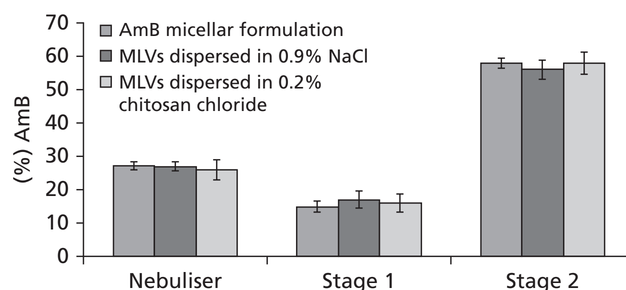

Figure 1 Deposition of amphotericin B in the two-stage impinger. Deposition of AmB in the two-stage impinger and remaining in the nebuliser following nebulisation of AmB deoxycholate micelles and multilamellar liposomes prepared by the thin-film method. AMB, amphotericin B; MLV, multilamellar vesicles. Each result is the mean (±SD) of three preparations

higher drug incorporation ($P < 0.05$) for liposomes hydrated with 0.2% chitosan chloride solution. To ensure the size of the formulations was less than 200 nm, liposomes were probe sonicated for 5 min (Table 1). The zeta potential of liposomes was positive when dispersed in 0.2% chitosan chloride and negative when dispersed in 0.9% sodium chloride (Table 1). The total aerosol mass outputs for the 0.9% sodium chloride and 0.2% chitosan chloride liposomes were 95.6 ± 1.2 and 93.8 ± 0.9%, respectively. Figure 1 shows the aerosol deposition pattern of the nebulised liposomal AmB, with the majority (56–58%) of the drug deposited in the lower stage of the TSI. This equates to 229.7 ± 9 and 188.6 ± 8 μg AmB in the lower stage for 0.2% chitosan chloride and 0.9% sodium chloride, respectively. During nebulisation, the size of the liposomes was reduced, with the smallest liposomes being collected in the lower stage of the TSI ($P < 0.05$; Table 2).

Amphotericin B liposomes prepared by the ethanol-based proliposomes method

Liposomes prepared in higher concentrations of chitosan chloride had the greatest median size ($P < 0.05$) even after sonication, and all the liposomal formulations were polydispersed (Table 3). Liposomes prepared by dispersing liposomes in 0.9% NaCl had a net negative charge, whilst dispersion in chitosan solutions produced liposomes having a net positive charge that was directly proportional to concentration (Table 3). The AmB-loading values for liposomes produced from proliposomes dispersed in 0.9% w/v sodium chloride and 0.2% w/v chitosan chloride were 66.7

Table 1 Properties of liposomes prepared by the thin-film method

Formulation	VMD before sonication µm ± SD (span ± SD)	Hydrodynamic diameter after sonication nm ± SD (PI ± SD)	Zeta potential after sonication mV ± SD	VMD of aerosol droplet µm ± SD (span ± SD)	AmB loading % ± SD
0.9% sodium chloride	4.19 ± 1.3 (1.35 ± 0.11)	181.7 ± 3.4 (0.292 ± 0.09)	−8.36 ± 2.8	3.43 ± 0.87 (1.28 ± 1.4)	46.7 ± 1.3
0.2% chitosan chloride	4.98 ± 1.5 (2.15 ± 0.54)	195.6 ± 1.6 (0.411 ± 0.12)	+11.5 ± 3.1	3.82 ± 1.66 (1.77 ± 0.92)	55.0 ± 1.6

AmB, amphotericin B; PI, polydispersity index; VMD, volume median diameter. Each result is the mean (±SD) of three preparations.

Table 2 Size characteristics of liposomes, produced by the thin-film method, delivered to the two-stage impinger and remaining in the nebulisers

Formulation	Hydrodynamic diameter in nebulizer nm ± SD (PI ± SD)	Hydrodynamic diameter in upper stage nm ± SD (PI ± SD)	Hydrodynamic diameter in lower stage nm ± SD (PI ± SD)
0.9% sodium chloride	169.3 ± 4.7 (0.344 ± 0.16)	152.7 ± 2.1 (0.385 ± 0.07)	137.4 ± 3.8 (0.320 ± 0.04)
0.2% chitosan chloride	178.6 ± 4.3 (0.378 ± 0.16)	164.6 ± 3.2 (0.464 ± 0.05)	146.7 ± 3.8 (0.382 ± 0.11)

PI, polydispersity index. Each result is the mean (±SD) of three preparations.

Table 3 Properties of liposomes generated by an ethanol-based proliposome method

Formulation	VMD before sonication µm ± SD (span ± SD)	Hydrodynamic diameter after sonication nm ± SD (PI ± SD)	Zeta-potential after sonication mV ± SD	AmB loading % ± SD
0.9% sodium chloride	2.98 ± 0.11 (1.09 ± 0.05)	172.2 ± 7.2 (0.385 ± 3.6)	−11.4 ± 5.9	66.7 ± 8.7
0.1% chitosan chloride	3.19 ± 0.13 (1.14 ± 0.08)	182.5 ± 5.1 (0.456 ± 0.09)	+15.1 ± 4.2	72.8 ± 6.1
0.2% chitosan chloride	4.51 ± 0.17 (1.87 ± 0.18)	205.0 ± 4.8 (0.552 ± 1.7)	+19.7 ± 1.5	78.2 ± 4.3
0.3% chitosan chloride	5.08 ± 0.21 (1.91 ± 0.13)	211.4 ± 3.3 (0.675 ± 1.4)	+22.9 ± 2.4	79.5 ± 5.7

AmB, amphotericin B; PI, polydispersity index; VMD, volume median diameter. Each result is the mean (±SD) of three preparations.

and 78.2%, respectively. These values were significantly higher ($P < 0.05$) than loadings for liposome formulations of similar composition prepared by the thin-film method (Table 1). Moreover, increasing the chitosan chloride concentration in the dispersion medium increased loading efficiency ($P < 0.05$; Table 3).

AmB proliposomes dispersed with 0.9% w/v sodium chloride or chitosan chloride solutions were sonicated for 5 min and filtered through 0.22 μm syringe filters. Resultant vesicles, viewed by TEM, showed small multilamellar structures (Figure 2a–d).

The aerosol droplet size of the nebulised formulations generated from proliposomes, as determined by laser diffraction, was in the range 2.5–3.3 μm (Table 4). There was a significant difference in the liposomal droplet size across groups ($P < 0.05$) and a post-hoc test revealed that the significant difference ($P < 0.05$) in the droplet size was between liposomes prepared with 0.9% sodium chloride and 0.3% chitosan chloride solutions. There was a statistically significant difference in span value between 0.9% sodium chloride liposomes and 0.1% chitosan chloride liposomes. Air-jet nebulisation reduced liposome size, with the smallest liposomes (range 135–148 nm) deposited in the lower stage of the TSI (Table 4). The mean hydrodynamic diameter of liposomes deposited in the lower impinger stage was largest for liposomes prepared in 0.3% chitosan chloride solution ($P < 0.05$).

The total aerosol mass outputs for the 0.9% sodium chloride and 0.1, 0.2 and 0.3% chitosan chloride liposomes was 94.3 ± 2.1, 96.1 ± 1.2, 95.6 ± 2.6 and 93.1 ± 2.6%, respectively.

After reaching 'dryness', when aerosol generation ceased, the majority (up to 60%) of the nebulised liposome-containing

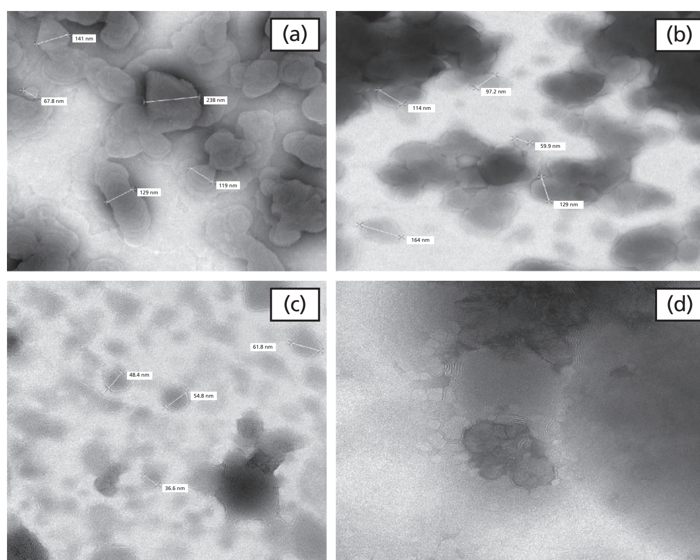

Figure 2 Transmission electron micrographs of sonicated proliposomes following hydration. Hydration in (a) 0.9% sodium chloride, (b) 0.1% chitosan chloride, (c) 0.2% chitosan chloride and (d) 0.3% chitosan chloride

Table 4 Size characteristics of liposomes generated by an ethanol-based proliposome method delivered to the two-stage impinger and remaining in the nebuliser

Formulation	Hydrodynamic diameter in nebulizer nm ± SD (PI ± SD)	Hydrodynamic diameter in upper stage nm ± SD (PI ± SD)	Hydrodynamic diameter in lower stage nm ± SD (PI ± SD)	VMD of aerosol droplets μm ± SD (span ± SD)
0.9% sodium chloride	164.5 ± 2.1 (0.352 ± 0.12)	153.7 ± 2.8 (0.366 ± 0.02)	135.2 ± 4.7 (0.422 ± 0.09)	2.46 ± 0.92 (2.15 ± 0.54)
0.1% chitosan chloride	163.4 ± 2.9 (0.452 ± 0.11)	148.3 ± 5.3 (0.292 ± 0.09)	136.5 ± 3.9 (0.384 ± 0.12)	2.67 ± 1.3 (1.35 ± 0.31)
0.2% chitosan chloride	191.8 ± 2.7 (0.643 ± 0.13)	168.5 ± 3.3 (0.689 ± 0.17)	138.2 ± 2.4 (0.511 ± 0.07)	2.96 ± 1.1 (1.92 ± 1.5)
0.3% chitosan chloride	189.8 ± 2.7 (0.643 ± 0.15)	168.5 ± 3.3 (0.489 ± 0.07)	148.2 ± 2.9 (0.451 ± 0.04)	3.28 ± 0.87 (1.80 ± 0.12)

PI, polydispersity index; VMD, volume median diameter. Each result is the mean (±SD) of three preparations.

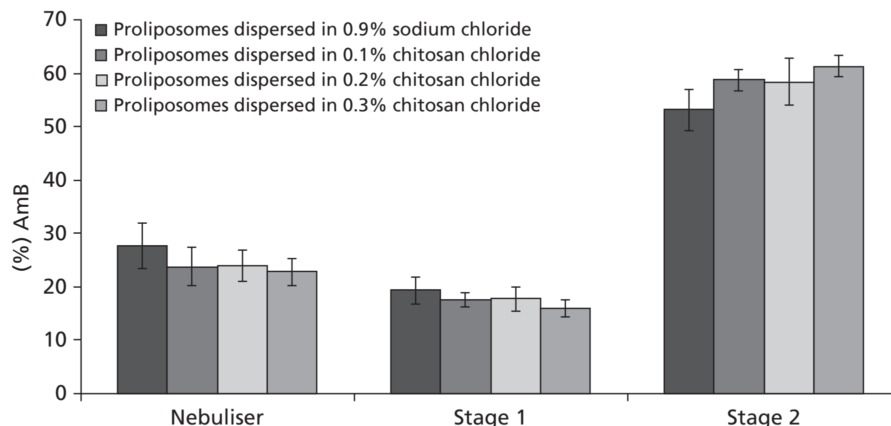

Figure 3 Deposition of amphotericin B for different dispersal agents. Deposition of AmB in two-stage impinger and remaining in the nebuliser following nebulisation of proliposomes dispersed in 0.9% sodium chloride and different concentrations of chitosan chloride. Each result is the mean (±SD) of three preparations. AMB, amphotericin B

AmB formulations were deposited in the lower stage of the TSI (Figure 3), with the greatest stage 2 deposition being achieved with the formulation containing 0.3% chitosan chloride. Up to 27% AmB remained in the nebuliser at the end of nebulisation. The chitosan-coated formulations had comparable AmB deposition (%) in the lower stage of the TSI to those dispersed in 0.9% sodium chloride (53.1 ± 2.6%), with deposition for 0.3, 0.2 and 0.1% being 61.3 ± 1.9, 58.4 ± 1.5 and 58.7 ± 2%, respectively ($P > 0.05$). This equates to 243.7 ± 11, 228.7 ± 7 and 213.7 ± 8 μg AmB in the lower stage for 0.3, 0.2 and 0.1% chitosan chloride and 177.1 ± 6 μg for proliposomes dispersed in 0.9% sodium chloride ($P < 0.05$). The greatest retention of AmB in the nebuliser chamber (27.6%) occurred for prolipo-somes dispersed in 0.9% NaCl ($P < 0.05$).

Antifungal activity of the amphotericin B formulations

Liposomes coated with chitosan chloride (0.1, 0.2 and 0.3%) and sodium chloride (0.9%) had fungal growth inhibition activities similar to micellar AmB. When the formulations were evaluated against two pathogenic yeast isolates, *C. albicans* and *C. tropicalis*, all had an MIC of 0.5 μg/ml.

Discussion

This study has identified a proliposome formulation, based on relatively low-cost materials, that is potentially applicable for the pulmonary delivery of an antifungal drug having systemic toxicity. The current proliposome formulation contains a small volume of the solvent DMSO. This is unlikely to present safety issues, since toxic effects may occur in humans if DMSO TD_{LO} exceeds 606 mg/kg for intravenous administration.[20] However, further development of this formulation approach may identify alternative solvent systems.

Amphotericin B entrapment in micelles and liposomes

In this study, an ethanol-based proliposome method was employed to produce AmB-containing liposomes as a potential delivery system for pulmonary administration. The small multilamellar liposomes produced by this methodology followed by sonication (Table 3) had a higher AmB-entrapment efficiency than liposomes of the same composition produced by the traditional thin-film method (Table 1). High entrapment in liposomes produced via proliposomes has previously been reported for a range of drugs including neomycin (65%), gentamicin (69%) adamantylamide dipeptide (87%), muramyl dipeptide (62%) and levonorgestrel (98%).[21,22] The reason for the enhanced incorporation of AmB in proliposome formulations of AmB has not been investigated, but may be related to changes in the packing behaviour of phospholipids and AmB in the liposomal bilayers, resulting from the presence of residual solvents.[23]

The ethanol-based proliposome preparation method, combined with the inclusion of chitosan in the formulation, gave a high AmB-loading efficiency, which in this study was maximal at 80% for the formulation containing 0.3% chitosan. This observation agrees with the enhanced entrapment and retention of the enzyme superoxide dismutase, achieved when liposomes are coated with chitosan to enhance mucoadhesive properties.[24] The mean liposome size, prior to and following sonication, was increased in the presence of chitosan chloride and increased further with higher concentrations of the polymer (Table 3). This can be attributed to more chitosan being present on the liposome surface.[25,26] The presence of more chitosan at the liposome surface leads to a greater median size, a larger positive charge density and higher zeta potential (Table 3), as previously described.[27] The charged polymer may also interact with phospholipid headgroups in the liposome bilayers, modifying packing behaviour and subsequently their susceptibility to size reduction by sonication.

Aerosolisation of amphotericin B formulations

The air-jet nebuliser used in this research (Pari LC Sprint) generates an aerosol with high mass output and droplets with a median size less than 3.3 μm (Table 4). This indicates the potential of these formulations for deep lung deposition, essential for effective local treatment of pulmonary fungal infections. For all the nebulised formulations studied, AmB

was deposited in a similar pattern within the TSI (Figures 1 and 3) with deposition predominantly in the lower stage, representing the fine particle fraction of the delivered aerosol. This was achieved by utilising small liposomes or micelles, combined with a nebuliser producing aerosols of small median size.[28] AmB output was in all cases lower than the total mass output. This effect has been reported previously for air-jet nebulisers, as there is preferential loss of solvent vapour during atomisation.[29] This increases the concentration of solutes or dispersed phase in the nebuliser chamber. In addition, there may also be retention of liposomes by physical structures, such as baffles within the device.[28] The total deposition of AmB in the lower stage of the impinger was in the order of 0.3% chitosan proliposomes >0.2% chitosan proliposomes = 0.2% chitosan multilamellar liposomes >0.1% chitosan proliposomes > deoxycholate micelles >0.9% sodium chloride multilamellar liposomes >0.9% sodium chloride proliposomes. For the proliposome formulations, the greatest retention of AmB in the nebuliser chamber occurred for liposomes prepared in 0.9% sodium chloride, and hence chitosan, possibly as a result of the charge imparted on the vesicles, enhanced delivery of the formulations from the nebuliser. The presence of the polymer may also modify the physicochemical properties of the fluids being nebulised. Such properties, in particular viscosity and surface tension, are known to influence the output from air-jet nebulisers.[30]

Following sonication and filtration, the liposomal median size was less than 200 nm. The mean size of liposomes deposited in the lower stage of TSI was less than 150 nm for all formulations, suggesting some deaggregation, size reduction or fractionation during air-jet nebulisation. This is in agreement with previous studies which showed that air-jet nebulisation has the effect of reducing the mean size of nebulised liposomal formulations in the micrometre size range.[3] Compared to samples prepared in 0.9% sodium chloride, liposomes with 0.3% chitosan solution deposited in both stages of the TSI had a larger mean diameter, indicative of the chitosan-coating of the vesicles, and perhaps reflecting the larger size of the aerosol generated from the 0.3% chitosan formulation. Moreover, all formulations containing chitosan produced greater deposition in stage 2 of the TSI and lower stage 1 deposition, suggesting that the presence of positive charge may decrease aggregation behaviour during aerosolisation.

Although the micelle formulations in this study had the greatest AmB loading (99.4%), the TSI nebulisation study revealed that all the ethanol-based proliposomes formulations studied are capable of delivering AmB at a higher dose to the lower stage of the TSI and hence are particularly therapeutically attractive. The potential for long-term stability also makes the proliposome formulation approach particularly attractive.[6]

Antifungal activity of amphotericin B formulations

The liposomal formulations in this study, with or without chitosan, exhibited identical antifungal activities against two *Candida* isolates. MIC values of the AmB containing liposomes were the same as micellar AmB, qualitatively and quantitatively comparable to proprietary Fungizone, which comprises deoxycholate micelles of AmB. These observations are consistent with previous studies that have shown that the minimal fungicidal concentrations and antifungal activities of a small unilamellar liposomal formulation of AmB against yeast and mould isolates are comparable to free AmB.[31,32] AmB in liposomal and free forms has previously been effectively nebulised to treat aspergillosis, but the function of pulmonary surfactant was inhibited by AmB deoxycholate.[15] Liposomes retained the fungicidal activity of AmB with the added advantage of reduced toxic side effects.[33] Incorporation of chitosan chloride in our formulations may enhance the liposome residence time at the targeted site and may thus improve the therapeutic antifungal effect of AmB, although this has yet to be determined.

Conclusions

Chitosan-coated liposomes, generated using an ethanol-based proliposome method, showed a high loading-efficiency of AmB and the in-vitro antifungal activity values were comparable to a deoxycholate micellar formulation. An air-jet nebuliser was used, which was capable of generating liposomal aerosols with a volume median diameter that would minimise the deposition of the droplets in the upper respiratory regions. In addition, the small size of the multilamellar vesicles combined with a small aerosol droplet size effectively delivered AmB to the lower stage of the TSI, predictive of deep-lung deposition and hence optimal activity against deep-lung fungal infections. Overall, this study suggests that chitosan-coated liposomes prepared from ethanol-based proliposomes are suitable as potential carriers, delivered via air-jet nebulisers, for pulmonary delivery of AmB.

Declarations

Conflict of interest

The Author(s) declare(s) that they have no conflicts of interest to disclose.

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