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Aerosolized liposome-based delivery of amphotericin B to alveolar macrophages

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Abstract

The present study was aimed at preparation, characterization, and performance evaluation of amphotericin B (Amp B)-loaded aerosolized liposomes for their selective presentation to lungs (alveolar macrophages), that being the densest site of Aspergillosis infection. Egg phosphatidylcholine (PC)- and cholesterol (Chol)-based liposomes were modified by coating them with alveolar macrophage-specific ligands (*O*-palmitoyl mannan, OPM, and *O*-polmitoyl pullulan, OPP). The prepared formulations were characterized in vitro for vesicle morphology, mean vesicle size, vesicle size distribution and percent drug entrapment. Pressurized packed systems based on preformed liposomal formulations in chlorofluorocarbon aerosol propellants were prepared. In vitro airways penetration efficiency of the liposomal aerosols was determined by percent dose reaching the peripheral airways, it was recorded 1.4–1.6 times lower as compared to plain drug solution-based aerosol. In vivo tissue distribution studies on albino rats suggested the preferential accumulation of OPM- and OPP-coated formulations in the lung macrophages. Higher lung drug concentration was recorded in case of ligand-anchored liposomal aerosols as compared to plain drug solution and plain liposome-based aerosols. The drug was estimated in the lung in high concentration even after 24 h. The drug-localization index calculated after 6 h was nearly 1.42-, 4.47-, and 4.16-fold, respectively, for plain, OPM-, and OPP-coated liposomal aerosols as compared to plain drug solution-based aerosols. These results suggest that the ligand anchored liposomal aerosols are not only effective in rapid attainment of high-drug concentration in lungs with high population of alveolar macrophages but also maintain the same over prolonged period of time. The significance of targeting potential of the developed systems was established.

Keywords: Aerosolized liposomes; Alveolar macrophages; Amphotericin B; OPM; OPP; Aspergillosis

1. Introduction

Aspergillus species are ubiquitous in the environment and are inevitably inhaled into the airways. Inhalation of *Aspergillus conidia* or mycelium fragments may result in colonization of the airways. In susceptible hosts, colonization may subsequently cause disease

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(Tomee and van der Werf, 2001). The fungus causes a variety of clinical syndromes in the lung (alveolar macrophages), ranging from aspergilloma in patients with lung cavities, to chronic-necrotizing aspergillosis in those who are mildly immunocompromised or have chronic lung disease. Invasive pulmonary aspergillosis (IPA) is a severe and commonly fatal disease that is seen in immunocompromised patients, while allergic bronchopulmonary aspergillosis is a hypersensitivity reaction to aspergillus antigens that mainly affects patients with asthma (Soubani and Chandrasekar, 2002). Aspergillus infections can involve almost every human tissue although predominantly present as a pulmonary infection. Most of the antifungal drugs presently in use, fail to penetrate macrophages, within which fungus lurk and that derives researchers to pursue delivery systems and their engineered versions in order to be therapeutically effective. Seemingly, potentiation of therapeutic effects while minimizing side effects entails for targeted delivery of antifungals to the infected macrophage tropics.

Amphotericin B (Amp B) is a drug of choice in systemic fungal infections (Walsh and Pizzo, 1990). The drug is also used in the treatment and management of visceral and mucocutaneous leishmaniasis, and in pulmonary aspergillosis (Sarosi, 1990; Denning and Stevens, 1990). Amp B manifests serious adverse complications related to dose-dependent acute and chronic toxicity. For diseases of microbial etiology, the intracellular localization of the pathogens necessitates the administration of relatively high doses of the cytotoxic drugs for the effective killing of the pathogens, thereby causing the side effects. The rational approach to the problem requires that the drug should be targeted to the macrophages in such a way that the interaction of the free drug with non-target tissues could be minimized. Maximum tolerated dose of Amp B is considerably low in mice. LD_{50} is 1.2 mg kg^{-1}, and doses higher than 1.6 mg kg^{-1} cause acute toxic reactions followed by cardiac–respiratory arrest (Lopez-Berestein et al., 1983). Treatment of disseminated fungal infections by liposomal Amp B results in a lower toxicity and significantly increased survival times (Brajtburg et al., 1990). It has been proposed that increased concentrations of drug in macrophages through passive liposomal uptake may improve its therapeutic index (Janknegt et al., 1992; De Marie et al., 1994). It was expected that ligand-mediated active targeting to the macrophages would significantly increase the rate and extent of macrophage accumulation of drug. This may reduce the required doses of liposomal Amp B in diseases like hepato-splenic fungal infections and leishmaniasis and in pulmonary aspergillosis associated with granulocytopenia. Mannose/fucose receptors expressed abundantly in liver, spleen, and alveolar macrophages have been most widely utilized for targeting bioactives to the macrophages (Vyas, 2000). The receptor facilitates endocytosis of glycoproteins terminated with mannose, fucose and glucosamine. In our laboratory, we have developed mannosylated liposomes for targeted delivery of Amp B to liver macrophages (Vyas et al., 2000).

Effective chemotherapy through drug targeting to alveolar macrophages can be practically implemented particularly in pulmonary aspergillosis using ligand-anchored liposomes. However, therapeutic applications of intravenously injected ligand-anchored liposomes have been limited due to several factors, such as leakage of their contents in plasma compartment before they reach the target tissue, rapid clearance from the blood stream, and their uptake by the macrophages of the liver and spleen. Pressurized packed or aerosolized liposomes for the pulmonary targeting of drugs have been well-documented (Farr et al., 1985, 1987; Vyas and Sakthivel, 1994; Vyas et al., 2004). These workers reported that the nebulization of liposomal dispersion allowed penetration to the peripheral region of the lung. Further, they established the concept of in situ liposome formation within the respiratory tract following deposition of inhaled microfine phospholipid aerosol delivered from solution-type pressure pack formulations. Aerosolized liposomal Amp B has also been reported for treatment of pulmonary fungal infections (Gilbert et al., 1992; Allen et al., 1994; Ruijgroka et al., 2001). Present study also deals with Amp B-entrapped ligand-appended aerosolized packed liposomes as possible means for direct targeting to the infected alveolar macrophages. The results of aerosolized plain drug solution, plain liposomes and ligand-appended liposomes are compared.

2. Materials and methods

2.1. Materials

Amphotericin B was obtained as a gift sample from M/s Ambalal Sarabhai Enterprises, Vadodara, In-

dia. Egg phosphatidylcholine (PC), cholesterol (Chol), stearylamine (SA), mannan, pullulan, sephadex G-50, Triton X-100 and phosphotungstic acid were purchased from Sigma, USA. Palmitoyl chloride was obtained from Fluka, Switzerland. Propellents P_{11} (trichlorofluoromethane) and P_{12} (dichlorodifluoromethane) were obtained from Himalaya Refrigeration Co. Ltd. (Bhopal, India). Chloroform and all other chemicals used were of pure analytical grade and obtained from Qualigens, Mumbai, India.

2.2. Preparation of ligand-coated MLVs containing Amp B

2.2.1. Preparation and optimization of MLVs containing Amp B

Multilamellar vesicles (MLVs) containing Amp B were prepared as described by Lopez-Berestein et al. (1983) with minor modifications. PC and Chol were dissolved in the minimum amount of chloroform and a methanolic solution (60 $\mu g\,ml^{-1}$) of Amp B was added to it. PC to Chol ratio (8:2 molar ratio) was kept constant, while Amp B content was varied at different mole percent ratio levels, i.e. (20, 16, 12, 8, 4, 2, and 1 mole% of the total lipids) in different preparations for determining optimum Amp B content. The organic solvent mixture was removed using a rotary flash evaporator under reduced pressure. The dried film was hydrated with 0.9% NaCl solution at $40 \pm 1\,^{\circ}C$ for 60 min and subsequently at room temperature for 6 h. The dispersion was centrifuged at 15,000 rpm for 4 h, and the pellet was resuspended in 0.9% NaCl solution.

The liposomal formulations were centrifuged through sephadex G-50 mini-column at 2000 rpm for 3 min for the separation of unentrapped drug. The liposomal fraction was added with minimum amount of Triton X-100 (0.1%, v/v), drug content was determined spectrophotometrically at 404 nm and percent drug entrapment was calculated. MLVs with optimum Amp B to lipid ratio were optimized for optimum PC to Chol ratio in terms of percent drug entrapment and toxicity towards erythrocytes. The MLVs with different PC to Chol ratios (9:1, 8:2, 7:3, 6:4, 5:5 molar ratios) were prepared. Amp B content, however, was kept constant at its optimum concentration level. The liposomes were evaluated for percent drug entrapment and toxicity to mammalian cells in terms of percent haemolysis. Percent haemolysis was determined by the method described by Mehta et al. (1984). Liposomal dispersions containing equivalent amount of Amp B (50 $\mu g\,ml^{-1}$) were incubated with 1.0 ml mammalian blood at $37 \pm 1\,^{\circ}C$ for 45 min, centrifuged at 10,000 rpm for 20 min, and haemoglobin released in the supernatant was measured spectrophotometrically at 550 nm (Mehta et al., 1984). For control, blood was similarly incubated with 0.9% NaCl solution. Blood was incubated with same volume of distilled water (100% haemolysis). From the haemoglobin released in supernatant, percent haemolysis in each case was computed and assessed. Percent haemolysis and percent drug entrapment were plotted against PC to Chol ratio, from which optimum PC to Chol ratio was determined.

2.2.2. Preparation of ligand-coated liposomes

Liposomes were coated with *O*-palmitoylated mannan (OPM) and *O*-palmitoylated pullulan (OPP) following the procedure developed in our laboratory with slight modifications (Vyas et al., 2000; Venkatesan and Vyas, 2000). *O*-palmitoylated mannan was synthesized from the yeast mannan by the process reported elsewhere in the literature (Hammerling and Westphal, 1967) and adopted and modified in our laboratory (Vyas et al., 2000). *O*-palmitoylated mannan, in brief was prepared by reacting mannan (1.0 g) in dry dimethyl formamide (DMF) at 60 °C with palmitoyl chloride (0.1 g) in DMF in the presence of dry pyridine (1.0 ml). The mixture was stirred at 60 °C for 6 h and slowly poured into absolute ethanol (100 ml) under vigorous stirring. The precipitate of OPM, thus obtained was collected and washed with 50 ml of absolute ethanol and 25.0 ml of dry diethyl ether, and dried in vacuo at $50 \pm 1\,^{\circ}C$ for 1 h. Similarly, OPP was synthesized by esterification of pullulan in DMF under catalytic conditions following the method of Sunamoto et al. (1985, 1987) with appropriate modifications in our laboratory (Venkatesan and Vyas, 2000).

Coating of Amp B-loaded MLVs with OPM was effected by incubating 1.0 ml of plain liposomal suspension with the dispersion of optimized amount of OPM (OPM:PC 0.15:1, w/w) in 0.9% (w/v) NaCl solution. The dispersion was stirred gently at room temperature for 4 h. Excessive, unbound polysaccharide was removed by spinning the resultant suspension through sephadex G-50 mini-column at 2000 rpm for 5 min (Vyas et al., 2000).

The coating of Amp B-loaded MLVs with OPP was effected by taking liposomal suspension and OPP in appropriate ratio (OPP:PC 4:6, w/w) and stirring the resultant dispersion for 6 h (Venkatesan and Vyas, 2000). Excessive polysaccharide was removed by centrifuging the resultant suspension at 15,000 rpm for 10–15 min. Due to higher density of the coated liposomes, they were settled, and a pellet was formed. The unbound OPP in the supernatant was removed.

For optimization of OPM:PC ratio, OPP:PC ratio and incubation time required for the effective coating, positively charged MLVs were prepared by incorporating stearylamine as one of the phospholipids at 0.5 mole% level of the total lipidic contents. These MLVs were incubated with varying amounts of OPM, i.e. w/w ratios based on PC weight (0.01:1, 0.05:1, 0.1:1, 0.15:1, 0.20:1, 0.25:1, and 0.5:1 (w/w) ratio) for 4 h and with varying amounts of OPP i.e. w/w ratios based on PC weight (1:9, 2:8, 3:7, 4:6, and 5:5 (w/w) ratio) for 1 h. After removing excessive unbound polysaccharide, specific electrical conductance of these liposomes was determined (Jaitely and Vyas, 1999). Conductivity was measured at an applied voltage of 10 mV using Systronics Conductivity Bridge 305 (India) and the measurements were made in μmhos. Specific electrical conductance was plotted against the OPM:PC ratio and OPP:PC ratio, respectively, and optimum OPM:PC ratio as well as OPP:PC ratio was determined from the plot as one, at or beyond which no further changes in the conductance were recorded. Similarly, to obtain optimum incubation time, formulations were incubated at optimum OPM concentration for different time intervals (0.5, 1.0, 1.5, 2.0, 2.5, 3.0, and 4.0 h), and at optimum OPP concentration for different time intervals (1, 2, 3, 4, 5, 6, and 7 h), and specific electrical conductance of these vesicles was recorded after separating the excessive, unbound polysaccharide. From the plot of specific electrical conductance against incubation time, optimum incubation time was determined to be one, at or beyond which no significant changes in the conductance were recorded.

2.3. Characterization of ligand-coated liposomes

Developed formulations were characterized prior to and after surface-ligand anchoring. The different liposomal formulations were evaluated for their shape and vesicle type both by phase contrast microscopy (Leitz-Biomed, Germany) and transmission electron microscopy (TEM) (JEM 1200, EX11, JEOL, Tokyo, Japan). Phosphotungstic acid (1%) was used as a negative stain. Carbon-coated samples were placed over a copper grid and subjected to TEM analysis. Vesicle size and size distribution were determined using laser diffraction-based particle size analyzer (CILAS, 1064, Marcoussis, France).

Percent drug entrapment was determined and expressed as the ratio of experimentally measured amount of drug in dispersion and initial amount used for entrapment. Vesicles (free of unentrapped drug) were lysed by adding 1.0 ml of 0.1% (v/v) Triton X-100, and liberated contents were analyzed for Amp B content spectrophotmetrically at 404 nm.

2.4. Preparation of aerosolized packed liposomes

Aerosols were prepared using already established procedure in our laboratory (Vyas and Sakthivel, 1994; Vyas et al., 2004). The solution phase pressure packs (30 ml volume) containing lipid/Amp B or Amp B solubilized in chlorofluorocarbon blend (P_{11}:P_{12}; 50:50, w/w) were prepared using a previously reported method with appropriate modification adopted as per laboratory setup (Farr et al., 1985). The required quantity of each ingredient was accurately weighed into a glass bottle and trichloromonofluoromethane (P_{11}) was filled in excess. Evaporation of P_{11} was permitted until the required weight was attained; this procedure also evacuates air foam within the bottle. The unit was hermetically sealed using a metering valve (fitted with a 20-mm ferrule) capable of delivering 25–100 μl of product per actuation, and the required quantity of dichlorodifluoromethane (P_{12}) was added, with the help of a pressure burette. Necessary facilities and equipments for aerosol filling operation were provided by M/s Rubicon Formulation Pvt. Ltd., Aurangabad, India.

2.5. Characterization of pressurized packed liposomes

The physicochemical characterization of various liposomal aerosol formulations was carried out for internal pressure, leak test, amount discharged per actuation, spray pattern, penetration efficiency, vesicle size, and percent drug entrapment.

2.5.1. Leak test and internal pressure

Leak test for the aerosol containers was conducted by immersing the containers into a water bath maintained at 35 ± 1 °C for 5 min (Farr et al., 1987). Internal pressure of the aerosol packs was measured using a pressure gauge (Sciarra and Cutie, 1987).

2.5.2. Aerosol valve discharge rate and spray pattern area

An aerosol product of known weight was allowed to discharge the contents for three actuations into a calibrated single stage liquid impinger, which was fabricated according to the design reported earlier (Farr et al., 1987; Vyas et al., 2004). Briefly, the apparatus fabricated consists of a 250-ml filtration flask containing a small open container partly filled with 5 ml aqueous receptor fluid, an intake tube with a smooth 90° bend protruded through the neck, with one end located just above the fluid surface and other end open for aerosol delivery. To ensure the delivery of a sufficient fraction of aerosolized dose to the receptor fluid, negative pressure was maintained by a tube connected to a vacuum pump. A 10-ml syringe was fitted to withdraw the samples for analysis (figure not shown). Aerosol valve discharge rate was determined with the weight difference of the aerosol container before and after three actuations (Sciarra and Cutie, 1987). Aliquots of receptor fluid from single stage liquid impinger were collected and average amount of Amp B delivered per actuation through different formulations was determined. The spray pattern area was based on the color produced on impingement of the spray, over a piece of paper pretreated with methylene blue–talc mixture, kept at 15 cm distance (Sciarra and Cutie, 1987).

2.5.3. Airways penetration efficiency

The term penetration efficiency from the valve is used to express relative efficiency of the developed aerosols measured as percentage of dose that eventually arrives at lungs. The apparatus and the method used were same as originally described by Kirk (1972) and adopted modified and fabricated using already established procedure in our laboratory (Vyas and Sakthivel, 1994; Vyas et al., 2004). Briefly, the apparatus was fabricated using glass tubes of 2.54-cm (1-in.) (Fig. 1). The upper part resembled, in shape and size, with a stylized human "trachea" and "bronchi." The two bronchi joined and proceeded to an adapter holding a membrane

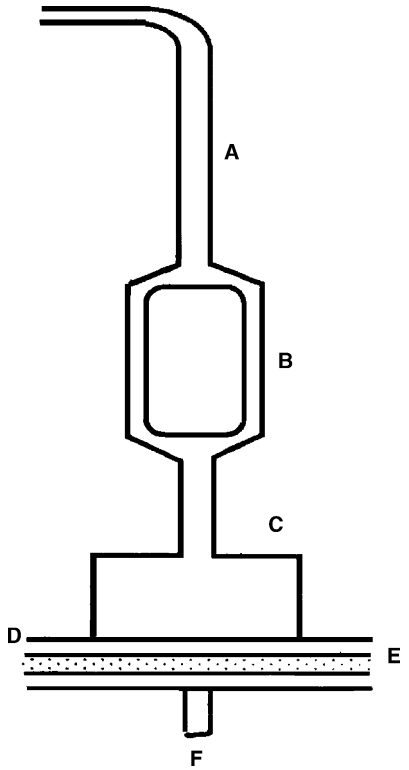

Fig. 1. Schematic diagram of airways penetration efficiency testing apparatus (Kirk, 1972); A, trachea; B, bronchi; C, adapter; D, filter holder; E, filter; F, respirator.

filter capable of retaining particles with a diameter of 1 μm or greater. To simulate the moist conditions of the respiratory tract, the whole of the glass tubing was lined with 3% agar gel, and air was drawn through the apparatus at a rate of 16 l min^{-1}, controlled by a flowmeter. The mouthpiece of the aerosol pack was kept at the throat of the apparatus and three actuations were then fired. The apparatus was disassembled and amount of Amp B deposited at different parts (filter, bronchi, and trachea) of the apparatus was determined.

2.5.4. Size characterization and percent drug capture of the liposomes formed after aerosolization of pressurized packed formulations

The vesicle size and the percent drug capture of liposomes formed following aerosolization of pressurized packed formulations were determined. The aerosolized products were characterized for spontaneous liposome formation by directing and delivering the aerosols gen-

erated from various pressurized packed liposome suspensions (plain- and ligand-appended liposomes) into a calibrated single-stage liquid impinger following three successive and continuous actuations (after vigorous shaking, flow rate 0.3 ml s^{-1}). Aliquots of receptor fluid from single stage liquid impinger were collected, which comprised of liposomes (after evaporation of propellants). The collected liposomes were studied for vesicle size and percent drug capture (Farr et al., 1987; Vyas and Sakthivel, 1994; Vyas et al., 2004).

2.6. In vivo studies

Albino rats of wistar origin (water and food ab libitum) were treated with different aerosolized packed liposomal formulations and the drug solution in aerosol form following the method reported in literature (Thomas et al., 1991; Myers et al., 1993). Formulations were studied for alveolar macrophage deposition and tissue distribution. Five groups of albino rats ($n = 6$) were used for the study. Rats were placed on nose-only exposure module. Animals were exposed to four actuations of aerosolized liposomal formulations and two actuations of aerosolized plain drug solution, as average amount of Amp B delivered per actuation was determined and found to be 35–40 μg in case of liposomal aerosols and 75–80 μg in case of plain drug solution aerosol. The first group of animals was served as control. Other groups were received plain drug solution aerosol and aerosolized plain neutral, and OPM- and OPP-coated liposomal formulations. Animals were sacrificed after 0.5, 1.0, 1.5, 3, 6, and 24 h. Blood samples were collected by cardiac puncture. Visceral organs (liver, spleen, lung, and kidney) of the dissected rats were removed and washed to remove any adhered debris, and dried using a tissue paper. The isolated organs were weighed separately, minced in pieces, and homogenized in 2.0 ml of phosphate buffer saline (pH 7.4) (Tissue Homogenizer, York, India). The homogenized tissues were deproteinized with 2.0 ml of methanol, kept in dark for 30 min and filtered. The resultant suspension was centrifuged for 20 min at 5000 rpm and filtered through 0.45 μm membrane filter in a 10 ml volumetric flask. Then the volume was made up to 10 ml with methanol and the drug content was measured using HPLC as discussed elsewhere (Van Etten et al., 1993). The serum was harvested from collected blood samples, deproteinized, and analyzed using the similar protocol as described earlier. The supernatants from successive extracts of an organ from each rat were pooled, and the drug content was determined. The amount of drug in each organ was calculated as micrograms of drug per milligram (wet mass) tissue, whilst the relative percent drug distributions were calculated and expressed as percent administered dose recovered from the organ as a whole.

Drug-localization index for each organ at different time intervals was calculated using formula given by Gupta and Huang (1989). Drug-localization index was calculated from the mean values of drug concentrations in organs achieved by test-delivery systems with respect to that achieved by plain drug solution.

2.7. Statistical analysis

The data were statistically processed to determine the level of significance. Standard deviation (S.D.) was calculated, and values are given as mean ± S.D. Student's *t*-test was used to compare mean values of different groups. Statistical significance was designated as $P < 0.05$.

3. Results and discussion

Targeting ligand anchored liposomes bearing Amp B to the lungs and specifically to the pulmonary alveolar macrophages by aerosolization, is a promising and feasible strategy for treating fungal infections. Alveolar macrophages form a first line of defense against microorganisms entering the lungs through the airways. In contrast to the interstitial macrophages in the lung, alveolar macrophages, which are located in the alveolar space have direct access to liposomes administered via the airways, for instance by intratracheal instillation, intranasal administration, or by application of aerosolized liposomes. The liposomes have been found to be considerably safe as carriers. The selected ligands for the present study were OPM and OPP, having specific affinity for macrophage mannose receptors. Liposomes prepared from PC as a constitutive lipid were optimized for various parameters. These include the molar ratio of Amp B to total lipid, ratio of Chol to PC at an optimized Amp B concentration and also the coating ratio and incubation time of the OPM and OPP.

Table 1
Types of structures formed with different molar ratios of Amp B to lipids

Amp B content[a]	Types of structures formed	Entrapment efficiency (%)
20	Comma-shaped ribbons and other unspecified structures	Not determined
16	Unspecified structures, ribbons, and some liposomal populations	Not determined
12	Distorted liposomes, ribbons, and some intact liposomes	Not determined
8	Mostly liposomes, some ribbons, and distorted liposomes	Not determined
4	Only liposomes of size range lower than 1.6–4.8 μm with average size 1.78 ± 0.42 μm	67.3 ± 1.6
2	Only liposomes of size range lower than 1.6–6.4 μm with average size 2.38 ± 0.89 μm	78.5 ± 1.6
1	Liposomes of size range 1.6–4.8 μm with average size 3.48 ± 0.97 μm	82.6 ± 1.4

Total lipid used, 50 mg; PC:Chol = 8:2 mole ratio for each formulation. Values are expressed as mean ± S.D. ($n = 6$).
[a] Percent molar ratio of Amp B to total lipids.

At higher concentrations (8–20 mole% of the total lipids) of Amp B, ribbons and unspecified structures were observed. However, as the concentration of Amp B was gradually lowered, relative numbers of ribbons and unspecified structures decreased, while number of liposomes increased (Table 1). It is speculated that the association of Amp B molecule with Chol may result in leaky membrane formation leading to lower entrapped volume and hence lower size of the vesicles. This hypothesis, however, needs confirmation from experimental studies. When molar concentration of Amp B was used at 2% of the total lipids, liposomal formulation was free of other undesired structures and average vesicle size measured was 2.38 ± 0.89 μm.

In another variation, with an increase in Chol concentration in liposomes at constant and optimized concentration of Amp B, distinctive changes in toxicity to the erythrocytes and percent entrapment of Amp B were recorded. On increasing the molar ratio of Chol:PC from 1:9 to 4:6, the percent haemolysis was recorded between 10.8 ± 2.05 and 1.0 ± 2.5% (Fig. 2). The mechanism behind this toxicity reduction of Amp B upon liposomal entrapment is still undefined. However, it seemed to be attributed to the more stable and compact configuration of bilayers and intercalation of Amp B in the bilayers. Probably, increased interaction of Amp B molecules with cellular Chol restricts their lateral partitioning with Chol present in the erythrocyte membranes. An increase in the concentration of Chol, however, resulted in a relatively low percent entrapment of Amp B (Fig. 2). It may, probably, be due to sterically favorable and hence, preferential accommodation of the Chol molecules within the bilayer assemblages. Optimum PC:Chol molar ratio was found to be 7:3, which formed spherical and multilamellar liposomes of mean vesicle size 2.56 ± 0.32 μm with maximum entrapment efficiency of 78.2 ± 1.3% and acceptably lower levels of toxicity towards erythrocytes (percent haemolysis = 1.4 ± 2.3%).

After optimizing the process parameters, PC liposomes were coated with OPM, which was characterized by IR analysis. Comparison of the infrared spectra of OPM with mannan revealed the presence of extra peaks due to C–C bond-deformation (2850 cm^{-1}) and C–H deformations (1490 cm^{-1}) arising from alkyl group in the product (spectra not shown). Peak that corresponds to C=O stretching vibrations expected at 1735 cm^{-1} appeared with a shift at 1710 cm^{-1}. It may be a consequence of intra-molecular hydrogen bond formation between carbonyl and hydroxyl groups, which suppressed the stretching force constant of C=O bond. C–O stretching vibrations appeared as a characteristic band in the range 1220–1190 cm^{-1}. All these peaks in the infrared spectra provided convincing evidences

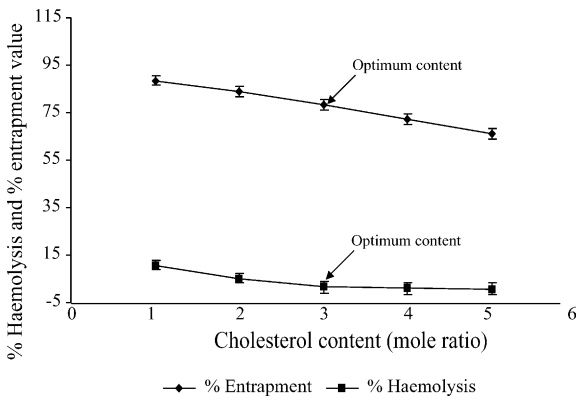

Fig. 2. Optimization of Chol content in MLVs containing Amp B in terms of percent haemolysis and percent entrapment (mean ± S.D.) ($n = 6$).

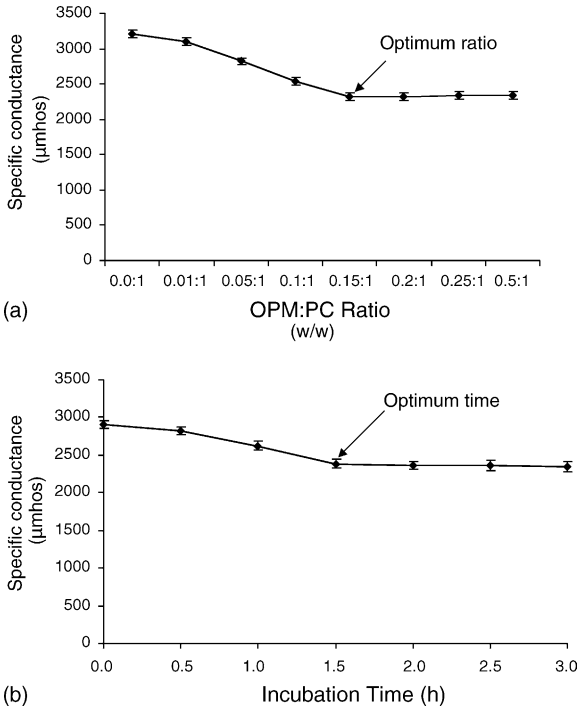

Fig. 3. Optimization of OPM:PC ratio and incubation time in terms of specific conductance at an applied voltage of 10 mV for the completion of coating process. Completion of coating was indicated by no further changes in specific conductance (denoted by arrow) (mean ± S.D.) ($n = 6$).

of the formation of an ester bond between mannan and *O*-palmitoyl anchor.

The OPM:PC ratio and incubation time were optimized by measuring the changes in electrical conductance of stearylamine containing liposomal suspension with varying concentrations of OPM as well as with variation in incubation time. A decrease in electrical conductance was observed from 3209.4 ± 52.1 to 2318.4 ± 51.2 μmhos, when the OPM:PC ratio was increased from 0.0:1 (w/w) to 0.15:1 (w/w) (Fig. 3a). It apparently relates to the extent of masking of surfacial charge (contributed by stearylamine) by OPM. Electrical conductance, however, remained nearly constant on further addition of OPM. This indicates no further charge-based interaction of the components of the bilayer and is suggestive of the completion of coating. The optimum OPM:PC ratio was found to be 0.15:1 (w/w), while optimum incubation time (the time that corresponds to completion of OPM coating under the experimental condition) recorded was 1.5 h (Fig. 3b), after which electrical conductance was remained fairly constant at 2386.0 ± 53.6 μmhos.

Comparison of the infrared spectra of OPP with pullulan revealed the presence of extra peaks due to C–C deformation (2848 cm^{-1}) and C–H deformation (1490 cm^{-1}) arising from alkyl group in the product (spectra not shown). Peak that corresponds to C=O stretching vibrations appeared to be shifted at 1709 cm^{-1}. C–O stretching vibrations appeared as a characteristic band in the range 1282 cm^{-1}. All the peaks in the IR spectra provided convincing evidences of the formation of OPP.

For optimization of OPP:PC ratio, a decrease in electrical conductance was observed from 2415.0 ± 52.4 to 1812.2 ± 53.5 μmhos, when OPP:PC ratio was increased from 0:10 (w/w) to 4:6 (w/w) (Fig. 4a). Electrical conductance, however, remained nearly constant on further increasing OPP:PC ratio. This indicates completion of coating. Optimum OPP:PC ratio was found to be 4:6 (w/w), while optimum incubation time recorded was 6 h, after which electrical conductance was remained fairly constant at 1460.3 ± 31.1 μmhos (Fig. 4b).

Plain liposomes and those anchored with ligands (OPM and OPP) were subjected to vesicle shape, size analysis, and percent drug entrapment measurements at appropriate steps. Morphologically, the vesicles were spherical in shape and multilamellar in nature. However, the ligand-appended liposomes were observed opaque, probably, due to distinct polysaccharide coating. Moreover, the ligand-appended liposomes were larger in size as compared to the uncoated liposomes. OPM-coated liposomes (PC:Chol:SA, 7:3:0.5 molar ratio), however, showed an average vesicle size of 3.15 ± 0.59 μm, while OPP-coated liposomes (PC:Chol:SA, 7:3:0.5 molar ratio) were of 3.23 ± 0.62 μm in size. Percent drug entrapment of OPM- and OPP-coated liposomes was 77.3 ± 2.5 and 76.8 ± 3.4%, respectively, revealing that OPM- and OPP-anchoring did not result in significant lowering of the percent drug entrapment as compared against 78.2 ± 1.3 recorded for plain extruded liposomes (Table 3). Preformed hydrated vesicles were used for anchoring of ligands and this may, presumably, be the reason for the insignificant changes recorded in the percent drug entrapment values. Relatively high entrapment of Amp B in the liposomes could be at-

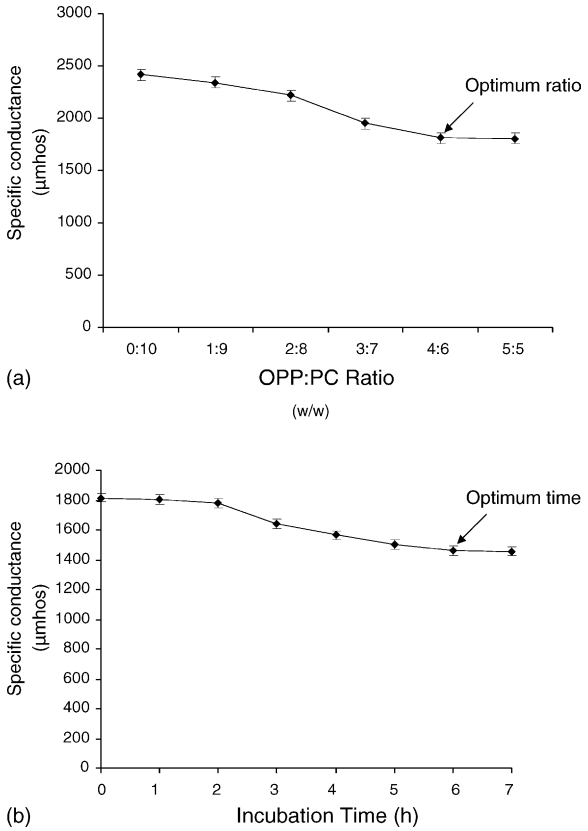

Fig. 4. Optimization of OPP:PC ratio and incubation time in terms of specific conductance at an applied voltage of 10 mV for the completion of coating process. Completion of coating was indicated by no further changes in specific conductance (denoted by arrow) (mean ± S.D.) ($n = 6$).

tributed to the lipophillic nature of the drug, since the entrapment was dependent upon lipid:aqueous phase ratio. Due to the lipophillic nature of the Amp B, it, probably, gets intercalated preferentially into the multilamellar vesicle lipid domains.

Different liposomal aerosols formulated were subjected to various physical and chemical characterization parameters as tabulated (Table 2). All formulated aerosols passed the leak test and the internal pressure was found to be almost constant (30 ± 4 psig, 1 psig = 6894.76 Pa) in all the containers. However, a small variation in internal pressure recorded may, presumably, be attributed to the viscosity and fractional vapour pressure of solutions with varied lipoidal composition. The aerosol valve discharge rate for liposomal aerosols was recorded to be in the range of 97–110 mg of suspension per actuation that was equivalent to 35–40 μg of Amp B. In the case of plain drug solution aerosol, the delivered dose was 108.4 mg per actuation, equivalent to 75–80 μg of Amp B. The spray area measured at a 15 cm distance for the liposomal aerosols was found in between 12.1 and 13.5 cm^2, whereas the plain drug solution aerosol produced an area of 14.9 ± 3.2 cm^2. This might be due to relatively viscous nature of the phospholipid-based aerosols. The more uniform spray pattern ensured uniform and optimum administration and eventually allowed it to reach the lung. Fig. 5 shows the relative amount of dose retained at various parts of the airways penetration efficiency testing apparatus. When plain drug solution aerosol was administered, a slightly higher ($P < 0.05$) amount of drug was measured on filter as compared to liposomal aerosols. This could be due to minimum retention of plain drug in upper airways. Unlike plain drug, in case of phospholipid-based aerosols application, the quantity of drug retained on filter was estimated to be low. This apparently relates to the retention of lipid-based fragments/vesicles in the airways (Fig. 1A and B), which may be due to relatively slow evaporation of propellant in the presence of phospholipids (Vyas et al., 2004). Photomicrograph PM 1 (Plate 1) shows aerosolized plain liposomes having mean vesicle size

Table 2
Characteristics of the various pressurized packed systems containing Amp B

Parameters	Drug solution	Plain liposomes PC:Chol (7:3)	OPM-coated liposomes PC:Chol:SA (7:3:0.5)	OPP-coated liposomes PC:Chol:SA (7:3:0.5)
Appearance	Homogeneous	Homogeneous	Homogeneous	Homogeneous
Leak test	Passed	Passed	Passed	Passed
Internal pressure (psig)	33.6 ± 1.4	32.8 ± 1.2	32.1 ± 1.6	31.6 ± 1.7
Discharge rate (mg per actuation)	108.4 ± 4.2	97.2 ± 6.3	102.3 ± 5.7	104.3 ± 6.2
Penetration efficiency (%)	51.2 ± 2.3	46.3 ± 3.2	31.2 ± 1.1	35.9 ± 1.6
Spray area at 15 cm (cm^2)	14.9 ± 3.2	13.5 ± 1.9	12.5 ± 2.4	12.1 ± 1.6

Values are expressed as mean ± S.D. ($n = 6$) (psig: pounds per square inch gauge; 1 psig = 6894.76 Pa in S.I. units).

Table 3
Vesicle size and entrapment efficiency of Amp B-loaded preformed and aerosolized liposomal systems

Liposome composition (mole ratio)	Vesicle size distribution			Mean vesicle size (μm)	% Entrapment
	Size range of ocular	Mean diameter (μm)	Average frequency		
Preformed liposomal systems					
PC:Chol (7:3) (plain uncoated)					
	0–1	0.72	23.33 ± 0.72	2.56 ± 0.32	78.2 ± 1.3
	1–2	2.16	34.66 ± 0.42		
	2–3	3.60	32.6 ± 0.54		
	3–4	5.04	9.33 ± 0.61		
PC:Chol:SA (7:3:0.5) (OPM-coated)					
	0–1	0.72	10.00 ± 0.98	3.15 ± 0.59	77.3 ± 2.5
	1–2	2.16	31.33 ± 1.05		
	2–3	3.60	38.00 ± 0.81		
	3–4	5.04	20.66 ± 1.29		
PC:Chol:SA (7:3:0.5) (OPP-coated)					
	0–1	0.72	8.33 ± 0.87	3.23 ± 0.62	76.8 ± 3.4
	1–2	2.16	30.66 ± 1.29		
	2–3	3.60	39.33 ± 0.81		
	3–4	5.04	21.66 ± 1.24		
Aerosolized pressure packed liposomes					
PC:Chol (7:3) (plain uncoated)					
	0–1	0.72	34.33 ± 1.24	2.27 ± 0.25	73.1 ± 2.5
	1–2	2.16	32.00 ± 1.69		
	2–3	3.60	25.00 ± 0.81		
	3–4	5.04	8.66 ± 2.05		
PC:Chol:SA (7:3:0.5) (OPM-coated)					
	0–1	0.72	11.66 ± 0.81	2.87 ± 0.47	72.7 ± 3.4
	1–2	2.16	41.33 ± 1.41		
	2–3	3.60	33.66 ± 2.05		
	3–4	5.04	13.33 ± 1.29		
PC:Chol:SA (7: 3:0.5) (OPP-coated)					
	0–1	0.72	8.33 ± 0.98	2.96 ± 0.58	71.9 ± 2.3
	1–2	2.16	42.66 ± 0.94		
	2–3	3.60	34.00 ± 1.24		
	3–4	5.04	15.00 ± 1.05		

Values are expressed as mean ± S.D. ($n = 6$).

of 2.27 ± 0.25 μm. PM 2 and 3 (Plates 2 and 3) show aerosolized OPM- and OPP-coated liposomes having mean vesicle size of 2.87 ± 0.47 and 2.96 ± 0.58 μm, respectively. The increase in mean vesicle size in case of aerosolized OPM- and OPP-coated liposomes as compared to aerosolized plain liposomes is an indication of coating, which can be distinguished by dark black boundary of the coated vesicles shown by arrow (PM 2 and 3) (Plates 2 and 3). A small but statistically significant decrease in the mean vesicle size was found in case of aerosolized liposomes, when compared with the preformed liposomal formulations. The percentage of entrapped drug was also found to be lower in case of aerosolized liposomes. The decrease in the mean vesicle size and percent drug entrapment of the liposomal aerosols as compared to preformed liposomes (Table 3) suggests that spontaneous reformation of liposomes could not be completed after aerosolization, resulting in the decrease in mean vesicle size and percent drug entrapment. However, the small changes observed were thought to be less important, because it is the aerosol droplet size, not the liposome size or

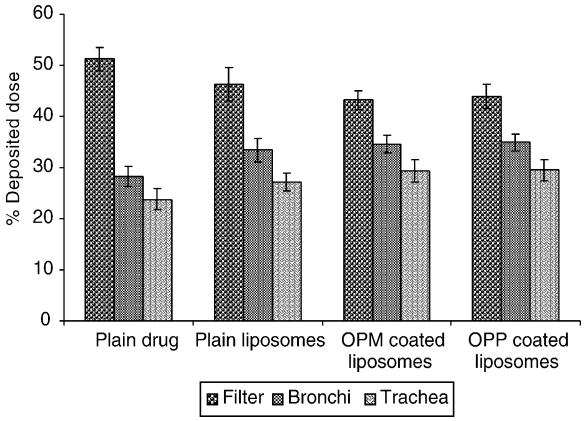

Fig. 5. Relative retention of deposited dose in various regions of the airways penetration efficiency testing apparatus from various formulated aerosolized systems (mean ± S.D.) ($n = 6$).

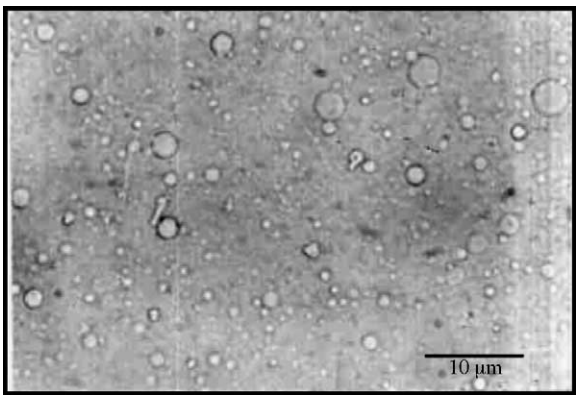

Plate 1. Aerosolized plain liposomes (450×).

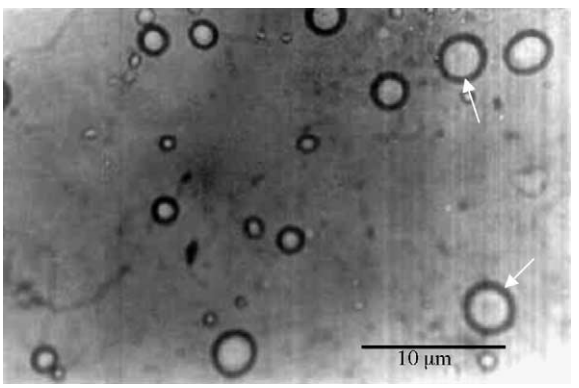

Plate 2. Aerosolized OPM-coated liposomes (1250×).

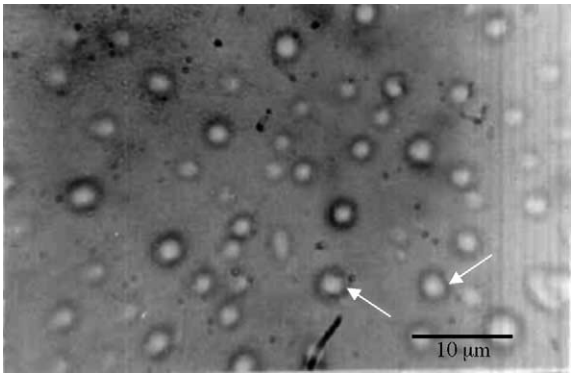

Plate 3. Aerosolized OPP-coated liposomes (1250×).

composition that determines the deposition of aerosol within the lung (macrophages). Thus, deposition is a function of the delivery device rather than the formulation (Farr et al., 1985; Vyas et al., 2004).

In vivo tissue distribution study of the developed aerosolized liposomal formulations was conducted on albino rats and compared with the controls (plain drug solution and plain liposomal aerosols). Table 4 shows the tissue distribution of Amp B after administration of different aerosolized liposomal formulations and plain drug solution. Both the developed liposomal formulations (OPM- and OPP-coated) showed greater accumulation in the lungs, when compared with the controls. In the case of free drug solution aerosol, only 36.42 ± 3.1% of the administered dose was found in the lungs at 0.5 h post-administration, and almost an equal amount was recovered in serum (28.37 ± 2.9%). The lungs, however, retained low amount of the administered dose with time whilst, and the serum concentration continuously increased reaching to a significant level. No drug was estimated in the lungs after 24 h. This may be attributed to the rapid absorption of plain drug solution to the systemic circulation from where it might be distributed and metabolized. It was due to pH of alveolar fluid, presence of lungs surface-active agent and large surface area of lungs alveoli. The pH may favour unionization of drug (pK_a 5.7 for acid and 10 for amine), however, due to continuous and fast absorption of unionized fraction through large alveolar surface, the solubility equilibrium of drug may be shifted to favour Amp B solubilization and absorption. Furthermore, lungs surfactant may play a role in solubilization of water insoluble drugs by forming soluble

Table 4
Organ distribution of Amp B following administration of various aerosolized formulations

Formulations	Time (h)	% Dose recovered in				
		Serum	Lung	Liver	Spleen	Kidney
Plain drug solution	0.5	28.37 ± 2.9	36.42 ± 3.1	7.89 ± 1.1	1.38 ± 0.12	ND
	1.0	34.97 ± 3.4	24.16 ± 2.2	13.76 ± 1.5	1.98 ± 0.14	1.76 ± 0.17
	1.5	39.65 ± 2.9	16.78 ± 1.4	16.92 ± 1.7	2.35 ± 0.38	2.63 ± 0.32
	3.0	30.16 ± 2.5	11.54 ± 0.92	12.13 ± 1.2	2.93 ± 0.23	4.69 ± 0.78
	6.0	19.16 ± 1.1	7.18 ± 1.2	7.45 ± 0.86	0.87 ± 1.1	7.36 ± 0.72
	24.0	5.12 ± 0.63	ND	2.31 ± 0.16	ND	1.13 ± 0.11
Plain uncoated liposomes (PC:Chol) (7:3)	0.5	19.36 ± 1.2	45.69 ± 3.9	4.12 ± 0.76	1.41 ± 0.05	ND
	1.0	24.18 ± 1.9	31.83 ± 2.7	15.86 ± 1.1	2.01 ± 0.09	1.87 ± 0.16
	1.5	30.18 ± 1.8	24.96 ± 2.8	22.73 ± 1.8	2.24 ± 0.17	2.84 ± 0.24
	3.0	23.17 ± 2.3	16.91 ± 1.3	25.13 ± 1.6	2.63 ± 0.23	5.21 ± 0.61
	6.0	15.46 ± 1.2	10.19 ± 2.1	16.14 ± 1.2	2.16 ± 0.13	7.98 ± 1.2
	24.0	4.83 ± 0.96	1.01 ± 0.03	3.01 ± 0.34	ND	0.98 ± 0.03
OPM-coated liposomes (PC:Chol:SA) (7:3:0.5)	0.5	8.62 ± 0.76	58.12 ± 4.9	2.68 ± 0.71	0.93 ± 0.03	ND
	1.0	17.92 ± 1.4	49.06 ± 4.2	7.63 ± 1.1	1.27 ± 0.11	1.03 ± 0.06
	1.5	23.17 ± 1.9	41.23 ± 3.8	11.02 ± 0.93	1.98 ± 0.11	1.62 ± 0.11
	3.0	18.67 ± 1.5	36.71 ± 3.1	13.17 ± 1.2	2.36 ± 0.09	2.73 ± 0.22
	6.0	11.96 ± 1.1	32.16 ± 2.9	8.92 ± 0.56	1.72 ± 0.02	3.98 ± 0.52
	24.0	4.12 ± 0.23	11.23 ± 0.9	1.96 ± 0.13	0.52 ± 0.01	1.09 ± 0.01
OPP-coated liposomes (PC:Chol:SA) (7:3:0.5)	0.5	10.56 ± 1.3	55.02 ± 4.5	4.16 ± 0.06	0.39 ± 0.08	0.17 ± 0.01
	1.0	17.94 ± 1.8	47.23 ± 5.1	7.89 ± 0.05	0.89 ± 0.05	0.69 ± 0.03
	1.5	22.86 ± 1.9	40.58 ± 5.9	11.22 ± 0.93	1.79 ± 0.06	1.12 ± 0.09
	3.0	19.32 ± 1.2	34.39 ± 4.2	14.69 ± 1.1	2.28 ± 0.09	2.23 ± 0.11
	6.0	16.59 ± 1.4	29.93 ± 3.3	10.92 ± 0.91	1.49 ± 0.03	2.98 ± 0.18
	24.0	4.01 ± 0.92	9.86 ± 1.6	3.13 ± 0.06	ND	1.15 ± 0.32

Values are expressed as mean ± S.D. ($n = 3$); ND, not detectable.

drug-surfactant complex. However, this needs further experimentals to test the solubilization behaviour of drugs in such systems in lungs. Plain liposomes (based on PC:Chol) showed an initial higher lung accumulation (45.69 ± 3.9% after 0.5 h). A fraction of so accumulated liposomes may be taken up by the alveolar macrophages, while major fraction of liposomes may be destabilized releasing free drug to follow absorption and distribution, as it occured in the case of plain drug. The concentration of drug thus estimated in lungs after 24 h was low (1.01 ± 0.03%). This may be attributed to the possible destabilization of plain liposomal vesicles in presence of Type II alveolar epithelial cells, which may utilize the liposomal phospholipids as precursors of lung surfactants leading to resultant drug leakage (Vyas and Khar, 2002). Therefore, clearance of drug from lungs may be attributed to the systemic absorption of released drug from destabilized liposomes. The mechanism by which Amp B gets from the lung to the systemic circulation is not known (Gilbert et al., 1992). Additionally, it is also probable that a fraction of liposomes significantly small in size may have an access to the circulation at alveolar blood capillary bed through fenestrae. However, they may get sequestered into RES predominant organs or may get accumulated into the sinusoidal spaces of such organs through extravasation via fenestrae. Therefore, it appears that for extravasation of any probable fraction of intact liposomes the organ-specific fenestrae may be equally responsible. The lung uptake of the ligand-appended liposomes was higher compared to the plain liposomes. The lung accumulation levels following administration of OPM-coated liposomes were 58.12 ± 4.9 and 32.16 ± 2.9% of the administered dose after 0.5 and

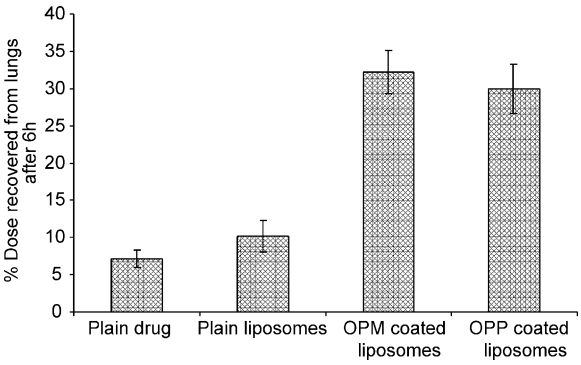

Fig. 6. Relative lung retention of various formulations after 6 h administration (mean ± S.D.) ($n = 3$).

6 h, respectively. Liposomes coated with OPP showed 55.02 ± 4.5 and 29.93 ± 3.3% accumulation in lungs at 0.5 and 6 h post-administration. Significantly higher Amp B concentrations (11.23 ± 0.9 and 9.86 ± 1.6%) were retained in the lungs even after 24 h following administration of OPM- and OPP-coated liposomes. The observed higher accumulation of these liposomes in alveolar macrophages may be due to their greater and selective affinity for liposomes coated with polysaccharide derivatives (e.g., amylopectin, palmitoyl conjugates of pullulan, pullulan phosphate) (Takada et al., 1984). Moreover, polysaccharide coat on the vesicles' surface offers protection against Type II alveolar epithelial cells which otherwise might have caused destabilization of plain uncoated liposomal vesicles. Fig. 6 shows the comparative percent drug recovered from lungs after 6 h administration of various formulations. The values are 1.42 times for plain liposomes, 4.47 times for OPM-coated liposomes and 4.16 times for OPP-coated liposomes, when compared to plain drug solution administered by aerosolization. The observed values suggest that the ligand-anchored liposomes are not only effective in rapid attainment of high drug concentration in alveolar macrophages (lungs) but could also maintain the concentration over a prolonged period of time, when compared against the free drug. This establishes the significance of the targeting potential of the developed systems.

The present study reveals that modification of the liposomal surface by the lung MPS-specific ligands like OPM and OPP improvised their stability and aerosolization of these ligand appended liposomes containing antifungal drug provides a possible means of drug targeting to alveolar macrophages. The technique circumvents and addresses the limitations, like possible leakage of the contents enroute to the target tissue, rapid clearance from the blood stream, and uptake by the macrophages of the liver and spleen, which are persistent with the intravenous administration. The lung accumulation of Amp B formulated in OPM-coated liposomes at 0.5 and 24 h after intravenous injection were found to be 9.1 ± 0.1 and 4.6 ± 0.02%, respectively (Vyas et al., 2000). However, in the present study, aerosolization of the same OPM-coated liposomes resulted in lung accumulation of 58.12 ± 4.9 and 11.23 ± 0.9% of administered Amp B after 0.5 and 24 h, respectively.

4. Conclusion

Results of this study suggest that encapsulation of antifungal drug (Amp B) in the liposomes, modification of the liposomal surface by anchoring lung macrophages-specific ligands and deposition into respiratory tract via aerosolization will certainly improve the chemotherapy against pulmonary aspergillosis. The strategy on the basis of in vivo performance appears to be promising. However, it is realized that the work should further be elaborated to study pharmacodynamics of the system(s), viz. macrophage activation profile and their combined role in eradication of *Aspergillus* infection.

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