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Design of liposomal aerosols for improved delivery of rifampicin to alveolar macrophages

S.P. Vyas*, M.E. Kannan, Sanyog Jain, V. Mishra, Paramjit Singh

Drug Delivery Research Laboratory, Department of Pharmaceutical Sciences, Dr. Harisingh Gour Vishwavidyalaya, Sagar, MP 470 003, India

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Abstract

The present study was aimed at preparation, characterization, and performance evaluation of rifampicin-loaded aerosolized liposomes for their selective presentation to alveolar macrophages, that being the most dense site of tuberculosis infection. Egg phosphatidylcholine (PC)- and cholesterol (Chol)-based liposomes were modified by imparting negative charge (using dicetylphosphate, DCP) or by coating them with alveolar macrophage-specific ligands (maleylated bovine serum albumin, MBSA; and *O*-steroyl amylopectin, *O*-SAP). The prepared formulations were characterized in vitro for vesicle morphology, mean vesicle size, and percent drug entrapment. Pressurized packed systems based on preformed liposomal formulations in chlorofluorocarbon aerosol propellants were prepared. In vitro airway penetration efficiency of the liposomal aerosols was determined by percent dose reaching the base of the lung, it was recorded 1.5–1.8 times higher as compared to plain drug solution-based aerosol. Percent viability of *Mycobacterium smegmatis* inside macrophages (in vitro) after administration of drug (in vivo) was in the range of 7–11% in the case of ligand-anchored liposomal aerosols, while it was recorded to be 45.7 and 31.6% in case of plain drug and plain neutral liposomal aerosol (based on PC:Chol)-treated macrophages. Results suggest the preferential accumulation of MBSA- and *O*-SAP-coated formulations in the lung macrophages, which was further reflected in the periodically monitored in vivo tissue distribution studies. Higher lung drug concentration was recorded in case of ligand-anchored liposomal aerosols and negatively charged liposomal aerosols (based on PC:Chol:DCP) as compared to plain drug 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 1.4- and 3.5-fold, respectively, for both ligand-appended liposome-based systems as compared to negatively charged and plain neutral liposome-based aerosolized systems. These results suggest that the ligand-anchored liposomal aerosols are not only effective in rapid attainment of high-drug concentration in lung 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.
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Keywords: Aerosolized liposomes; Alveolar macrophages; Rifampicin; MBSA; *O*-SAP; Tuberculosis

1. Introduction

Tuberculosis is one of the major infectious diseases worldwide and its incidence is increasing particularly in association with AIDS pandemic. Among various forms of tuberculosis, pulmonary tuberculosis is most commonly characterized by the involvement of

* Corresponding author. Tel.: +91-7582-226525; fax: +91-7582-226525.

E-mail address: spvyas@sancharnet.in (S.P. Vyas).

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alveolar macrophages harboring a large number of tubercle bacilli. The tubercle bacilli penetrate inside the macrophages subsequently to protectorate through intracellular harboring. The bacilli secrete molecules that prevent phagosome–lysosome fusion. Moreover, due to very hydrophobic waxy cell wall, bacilli are resistant to digestion by lysosomal enzymes and hence resist the killing effects of macrophage (Bermudez, 1994). Most of the anti-tubercular drugs presently in use, fail to penetrate macrophages within which bacilli lurk and that derives researchers to persue 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 antibacterials to the infected macrophage tropics.

Rifampicin is the first choice drug in the treatment of tuberculosis but requires a high-dose drug treatment over a period of 4–6 months. The causative organism is known to develop resistance with conventional delivery systems as serum levels were found to fluctuate below minimum inhibitory concentrations in clinical investigations. Rifampicin also has various side effects at dose levels administered in long-term clinical therapeutics, such as immunological disturbances, rheumatoid, or lupoid syndromes, allergic rashes, eosinophilia, leukopenia, and other hepatotoxic manifestations (Mandell and Sande, 1985). Several novel delivery devices bearing rifampicin have been characterized by Vyas and co-workers in order to maximize the therapeutic indices and minimize the associated side and toxic effects (Pandey et al., 1991; Jain and Vyas, 1995; Nakhare and Vyas, 1995a,b, 1997).

Liposomes have been advocated as carrier to deliver the antibacterials to the pre-selected targets. Pressurized packed liposomes for the pulmonary targeting of drugs have been well documented (Farr et al., 1985, 1987; Vyas and Sakthivel, 1994). 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 disposition of inhaled microfine phospholipid aerosol delivered from solution-type pressure pack formulations. Liposomes have also emerged as a carrier cargo for cell- and organ-specific targeting using ligands, including proteins, peptides, polysaccharides, glycolipids, glycoproteins, lectins, and anti-target monoclonal antibodies (Ostro, 1987). Effective chemotherapy through drug targeting to alveolar macrophages can be practically implemented particularly in pulmonary tuberculosis 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. In the present study, an attempt has been made to combine the concept of aerosolization and ligand-mediated targeting to alveolar macrophages. Macrophage-specific ligands can be used as a sensing module appended to liposomes. Present study deals with rifampicin-entrapped ligand-appended aerosolized packed liposomes as possible means for direct targeting to the infected alveolar macrophages. The results of plain, ligand appended, and their aerosolized versions are compared.

2. Materials and methods

2.1. Materials

Drug rifampicin was a kind gift from M/s. Kerala State Drugs and Pharmaceuticals, Alappuzha, Kerala (India). Egg phosphatidylcholine (PC), dicetylphosphate (DCP), cholesterol (Chol), maleylated bovine serum albumin (MBSA), amylopectin, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC), and sephadex G-50 were purchased from Sigma Chemical Co., USA, and used as supplied. Propellents P_{11} (trichlorofluoromethane) and P_{12} (dichlorodifluoromethane) were obtained from Himalaya Refrigeration Co. (Bhopal, MP, India). Bacterium *Mycobacterium smegmatis* was procured from Institute of Microbial Technology, Chandigarh, India. Chloroform and all other chemicals used were of pure analytical grade and obtained from Qualigens (a division of Glaxo India Ltd.).

2.2. Preparation of multilamellar vesicles by lipid film hydration method

Neutral liposomes were prepared by cast film method. Constitutive lipids (PC:Chol; 7:3 molar ratio)

were dissolved in minimum volume of chloroform. Known amount of drug (1 mg/ml) dissolved in organic solvent was added to the lipid solution. Organic solvent was slowly removed under reduced pressure, using a Buchi rotary flash evaporator, so that a thin film of dry lipid was deposited on the inner wall of the flask. The thin lipid film was dispersed in phosphate buffer saline (PBS, 0.01 M, pH 7.4) to give a final phospholipid concentration of 100 μmol/ml by mechanical agitation using a wrist action shaker for 3 h. The swollen lipid film was hydrated at 40 ± 2 °C. Unentrapped drug was removed by passing the liposomal suspension through sephadex G-50 mini column and centrifugation at 3000 rpm for 3 min (New, 1990). Negatively charged liposomes were prepared by incorporating DCP in the formulation at a molar ratio of 0.1:10 with respect to total lipids.

2.3. Preparation of ligand-coated liposomes

Neutral liposomes were coated with phospholipid-tailed MBSA (PE–MBSA conjugate) (Mukhopadhyay et al., 1989; Chaudhuri et al., 1989) and *O*-steroyl amylopectin (*O*-SAP) (Takada et al., 1984). PE–MBSA conjugate was synthesized using EDC-mediated coupling reaction. Conjugate was purified from excess reactants and other small molecules by Sephadex G-50 gel filtration (Chaudhuri et al., 1989; Hermanson, 1996). Similarly, *O*-SAP was synthesized by esterification of amylopectin in dimethyl formamide under catalytic conditions following the method of Sunamoto and co-workers with appropriate modifications (Sunamoto et al., 1985). Uncoated formulations were incubated with phospholipid-tailed MBSA and *O*-SAP for coating, and coated liposomes were further evaluated. Coating parameters were optimized by measuring the change in zeta potential of the dispersion (Aplex 35, France). Coating ratio of ligand with respect to total lipid was varied. Similarly, at an optimized lipid to ligand ratio the incubation time periods were varied. Surface potential of different formulations was calculated using Helmholtz–Smoluchowski's equation (Adamson, 1967).

2.4. In vitro characterization

Various liposomal formulations were evaluated for vesicle shape and morphology using transmission electron microscopy (TEM). Phosphotungustic acid (1%) was used as a negative stain. Samples were placed over a copper grid and subjected to TEM analysis (JEM-200 CX, JEOL, Tokyo, Japan). Vesicle size and size distribution were determined using particle size analyzer CILAS 1064 (France), which works on a laser diffraction principle.

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 rifampicin using HPLC method using a mobile phase of 0.01 M acetate buffer (pH 7.0):acetonitrile (1:1) at a detection wavelength of 340 nm (Jamaluddin et al., 1990).

2.5. Preparation of aerosolized packed liposomes

Aerosols were prepared from 10 ml of liposomal formulations using already established procedure in our laboratory (Vyas and Sakthivel, 1994). The solution phase pressure packs (30 ml volume) containing lipid/rifampicin or rifampicin solubilized in cholorofluorocarbon 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 an excess. Evaporation of P_{11} was permitted until the required weight attained; this procedure also evacuates air foam within the bottle. The unit was hermetically sealed using a metering valve, and the required quantity of dichlorodifluoromethane (P_{12}) was added, with the help of a pressure burette.

2.6. Characterization of pressurized pack liposomes

The pressurized containers were studied for spontaneous liposome formation utilizing a single piece liquid impinger (Vyas and Sakthivel, 1994) and the aliquots withdrawn from it were studied for vesicle size, percent entrapment, and other parameters. The

characterization of various liposomal aerosol formulations was carried out for internal pressure, leak test, amount discharged per actuation, spray pattern, and penetration efficiency.

2.6.1. Leak test and internal pressure

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

2.6.2. 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 (Farr et al., 1987). Aerosol valve discharge rate was determined with the weight difference of the aerosol container before and after three actuations. Aliquots of receptor fluid from single stage liquid impinger were collected and average amount of drug (rifampicin) delivered per actuation through different formulations was determined (Sciarra and Cutie, 1987).

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).

2.6.3. Airways penetration efficiency

The term 'penetration efficiency from the valve' is used to express relative efficiency of the nebulizer measured as percentage of dose that eventually arrives at lungs. The apparatus and the method used were as described by Kirk (1972). Briefly, the apparatus was fabricated using glass tubes (Fig. 1). The upper part resembled, in shape and size, the human trachea (12.5 cm) and bronchi. The two bronchi were lengthened to simulate the conditions and joined to an adapter holding a sintered glass filter capable of retaining particles larger than 1 μm. To simulate the conditions of the respiratory tract, the whole of the system was coated with 3% agar gel, and air was drawn through the apparatus at a rate of 14 cycles/min.

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 rifampicin deposited at different part of the apparatus was determined.

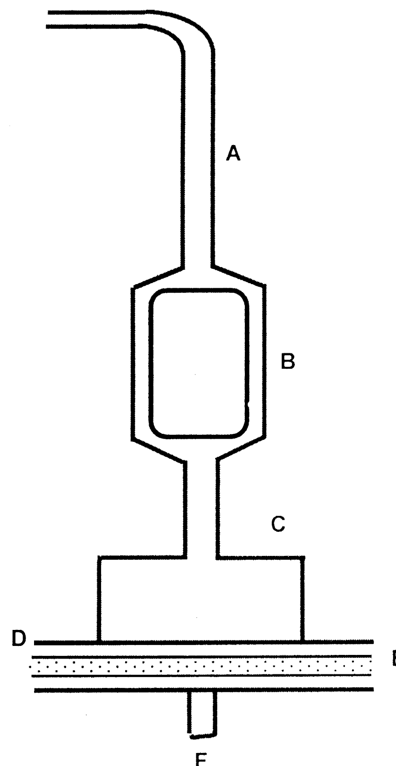

Fig. 1. Schematic diagram of airway penetration efficiency testing apparatus (Kirk, 1972): A, trachea; B, bronchi; C, adapter; D, filter holder; E, filter; F, respirator.

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

The vesicle size and the percent capture of liposomes formed following aerosolization of pressurized packed formulations were determined. The aerosolized products were characterized by directing and delivering the aerosols generated from various pressurized packed liposome suspensions (i.e. neutral (PC:Chol), negatively charged (PC:Chol:DCP), and ligand-appended liposomes) into a calibrated single stage liquid impinger (three actuations) (Farr et al., 1987). 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.

2.7. In vitro phagocytic activity and determination of percent viability of bacilli inside the macrophage monolayers (in vitro) after administration of rifampicin in vivo

Albino rats of Wistar origin were treated with different aerosolized packed liposomal formulations and the drug solution in aerosol form following the method reported in literature (Myers et al., 1993; Thomas et al., 1991). Six groups of albino rats (×6) were used to collect alveolar macrophages. Rats were placed on nose-only exposure module. Animals were exposed to six actuations of aerosolized liposomal formulations and three actuations of aerosolized plain drug solution, as average amount of rifampicin delivered per actuation was determined and found to be 45–55 μg in case of liposomal aerosols and 100 μg in case of plain drug aerosol. The first group of animals served as control. Other groups received plain drug solution aerosol and aerosolized plain neutral (PC:Chol), negatively charged (PC:Chol:DCP), and MBSA- and *O*-SAP-coated liposomal formulations. The alveolar macrophages (AMϕ) were harvested from whole lung lavage of animals following the method reported by Rothi and Harris (1986). Briefly, rats were anaesthetized by intraperitoneal injection of sodium pentobarbital (0.2 gm/kg body weight). The trachea was cannulated and the lungs lavaged 10 times with 10 ml aliquots of sterile isotonic saline. Lavage cell suspensions were centrifuged at $500 \times g$ for 5 min at 4 °C and the resulting pelleted cells were resuspended in the culture medium. The cells were enumerated with a hemocytometer and cell viability was measured via trypan blue exclusion. One hundred microliters of aliquot containing 1×10^6 cells/ml with more than 95% viability level was added onto the bottom of plastic culture tubes and the cells were allowed to adhere on the glass surface at room temperature for 2 h prior to use while non-adherent cells were removed by repeated rinsing. The AMϕ-enriched monolayers were incubated at 37 °C, in a humidified atmosphere for 24 h. The model bacilli (*M. smegmatis*) (2×10^6 CFU) were exposed to macrophage monolayers for 72 h at 37 ± 1 °C. The bacilli that were not phagocytosed by macrophages were washed off by decantation and washing of the coverslips. One set of coverslips for all treatments was fixed in 2.5% glutaraldehyde and stained for counting acid-fast stainable bacteria inside the macrophages by Ziel Neelson's technique. A total of 100 macrophages were scored and the numbers of acid-fast stainable bacteria per 100 macrophages were calculated. For the assessment of viable bacteria inside the macrophages, fluorescein diacetate (FDA)/ethidium bromide (EB) staining procedure was adopted. This method was based on the FDA/EB staining of bacilli. The viable bacilli convert non-fluorescent FDA to fluorescent fluorescein, which accumulates within intact bacilli and make them fluoresces as green. The dead cells take up EB (due to defective membrane) and appear as orange-red under incident UV light. The number of green fluorescing bacilli/macrophage was recorded. This count was divided by the total acid-fast bacilli/macrophage observed before to give the percentage of viability, determined microscopically.

2.8. In vivo studies

The developed liposomal aerosols were studied for alveolar macrophage deposition and tissue distribution. In vivo organ distribution studies were performed on male albino rats of Wistar origin (water and food ab libitum) following administration of various aerosolized products. Animals were exposed to various aerosolized formulations as described previously and were sacrificed after 0.5, 1, 2, 4, 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 into pieces, and homogenized in 2.0 ml of phosphate buffer (Tissue Homogenizer, York, India). The homogenized tissues were deproteinized with 2.0 ml of methanol and kept in dark for 30 min and filtered. To the filtrate, 5.0 ml of extraction fluid (butanol:hexane::4:1) was added with vigorous shaking and then centrifuged (5000 rpm, 30 min). The organic supernatant was separated and drug content was measured using HPLC as discussed elsewhere (Jamaluddin et al., 1990). The serum was harvested from collected blood samples, deproteinized, and extracted 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. Similarly, a group of six rats was exposed to aerosolized plain drug solution, and serum drug concentration and organ distribution profiles were monitored. The amount of drug in each organ was calculated as micrograms of drug per milligram (wet mass) tissue, whilst the relative percent 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 has been calculated from the mean values of drug concentrations in organs with respect to that achieved by plain drug solutions.

2.9. Statistical evaluation

The data were statistically processed to determine the level of significance. Using the two-sided F-ratio test and t-test, the means of two data sets were compared. The significance was evaluated at 1% probability level ($P < 0.01$ denoting significance).

3. Results and discussion

Targeting ligand-anchored liposomes bearing rifampicin to the lungs and specifically, to the pulmonary alveolar macrophages by aerosolization, is a promising and feasible strategy for treating mycobacterial infections. The selected ligands for the present study were MBSA and *O*-SAP. MBSA is a ligand, which has specific affinity for macrophage scavenger receptors. Considering its exquisite specificity and abundance of its expression, the macrophage scavenger receptor and ligand system was chosen for targeted rifampicin delivery to macrophages (Mukhopadhyay et al., 1989). Another ligand *O*-SAP was selected considering its greater affinity towards alveolar macrophages. The alveolar macrophages have been identified to selectively uptake *O*-SAP-coated liposomes (Deol and Khullar, 1997).

Initial experiments were conducted to optimize the procedure of ligand anchoring. The parameters studied include ligand:lipid ratio and optimum incubation time. The value of zeta potential varied marginally on addition of anionic ligand, MBSA, up to 5:1 lipid:ligand weight ratio (Fig. 2a). On further addition of MBSA, especially beyond the optimum lipid:ligand ratio (5:1), a significant change in the zeta potential occurred, however, it was with negative deviation. It indicates that at an optimal 5:1 weight ratio of lipid:ligand, the integration of phospholipid-tailed MBSA with the phospholipid bilayer membrane occurs at saturation level while further addition results in to an excess of free phospholipid-tailed MBSA, which contributes to an increase in the surface potential values. The incubation time for coating was optimized by measuring the surface potential of the liposomal formulation at the optimum MBSA to lipid ratio at different incubation time periods, viz. 0, 1, 2, 3, 4, 6, 8, 12, and up to 24 h (Fig. 2b). The surface potential values fell down steeply from their initial values. The decrease in the surface potential values may be attributed to the charge quenching of surface-associated free MBSA. With longer incubation time, the amount of residual free MBSA decreased as measured in terms of surface potential. However, the change in surface potential after 12 h was negligible to nil. This shows that at the end of 12 h, the interaction and integration of added MBSA could have been completed. The 12-h incubation time was considered as the optimum coating incubation time.

As in the case of MBSA, the surface potential values were used to optimize *O*-SAP:total lipid ratio. The liposomal dispersion was stirred with different *O*-SAP:total lipid ratio for different incubation periods. The *O*-SAP:lipid ratio 1:3 (w/w) was considered as optimum for coating multilamellar vesicles with *O*-SAP as measured surface potential values were found to be least variant after 6 h of incubation (Fig. 3a and b).

Morphologically, the vesicles were spherical in shape and multilamellar in nature. However, the ligand-appended liposomes were observed opaque probably due to a distinct protein polysaccharide coating. The vesicle size of the plain neutral liposomes (PC:Chol) compares well with the negatively charged liposomes composed of PC:Chol:DCP (i.e. 2.32 ± 0.48 μm and 2.50 ± 0.54 μm, respectively). Moreover, the ligand-appended liposomes were larger in size as compared to the uncoated liposomes. MBSA-coated liposomes, however, showed an average vesicle size

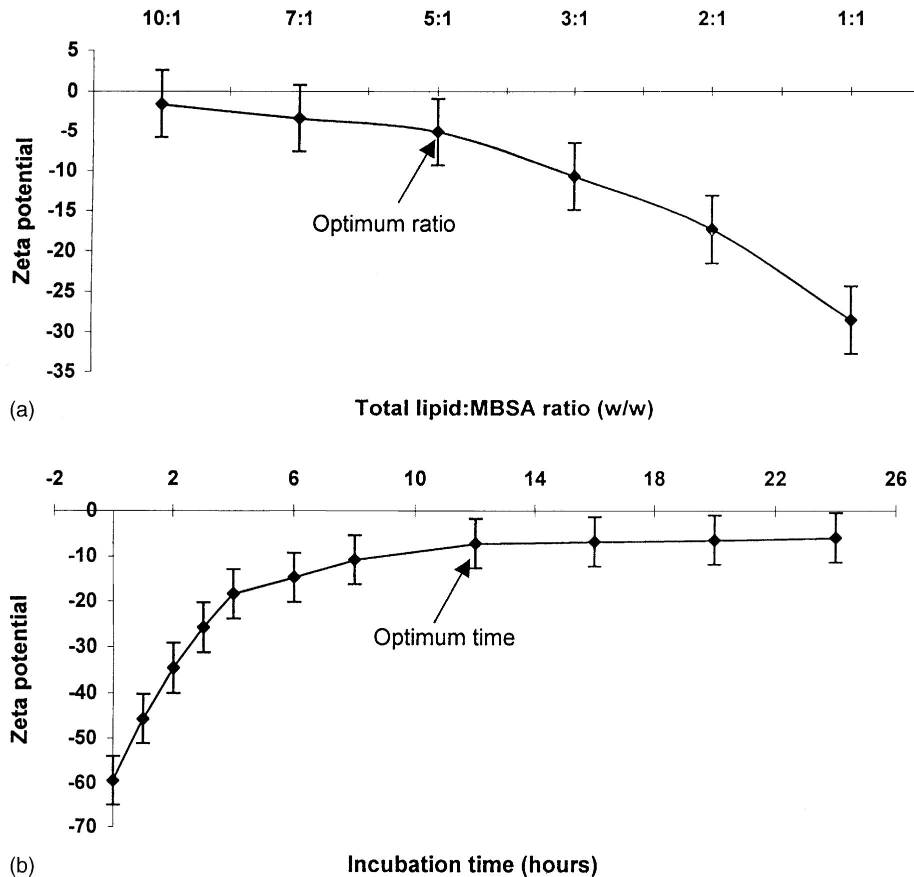

Fig. 2. Optimization of MBSA coating process to liposomes. (a) Optimization of total lipid:MBSA ratio and (b) optimization of incubation time.

of 3.64 ± 0.65 μm, while *O*-SAP-coated vesicles were of 3.85 ± 0.59 μm in size. Relatively high entrapment (47.4 ± 2.7%) of rifampicin in the liposomes could be attributed to the lipophilic nature of the drug since the entrapment is dependent upon lipid:aqueous phase ratio. Due to the lipophilic nature of the rifampicin, it probably gets intercalated preferentially into the multilamellar vesicle lipid domains. A marginal increase in the percent drug entrapment was measured (49.8 ± 3.1%) on incorporation of DCP as a charge-imparting lipid. This might be attributed to the ion pairing of the positive moiety of rifampicin with the negatively charged DCP and probably due to their favored partitioning in the lipid bilayer. The level of drug entrapment in the case of coated liposomes was more or less similar to that of plain liposomes.

Different liposomal aerosols formulated were subjected to various mechanical and chemical characterization parameters as tabulated (Table 1). All formulated aerosols passed the leak test and the internal pressure was found to be almost constant (31 ± 4 psig) in all the containers. However, a small variation in internal pressure recorded may presumably be attributed to the viscosity and fractional vapor pressure of solutions with varied lipoidal composition. The aerosol valve discharged rate for liposomal aerosols was found to be in the range of 101–113 mg of suspension per actuation, that was equivalent to 45–55 μg of rifampicin. In the case of plain drug solution aerosol the delivered dose was 113.6 mg per actuation, equivalent to 100 μg of rifampicin. The spray area measured at a 15 cm distance for the liposomal aerosols

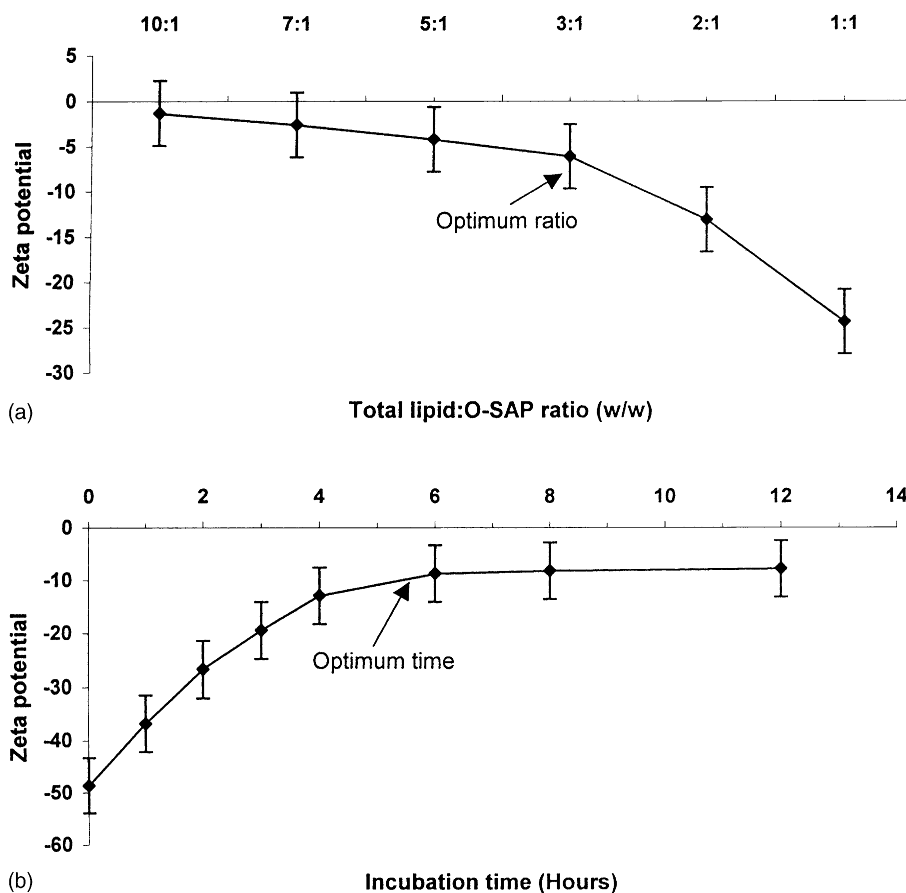

Fig. 3. Optimization of *O*-SAP coating process to liposomes. (a) Optimization of total lipid: *O*-SAP ratio and (b) optimization of incubation time.

was found in between 12.6 and 13.8 cm^2, whereas the plain drug solution aerosol produced an area of 15.2 ± 2.6 cm^2. This might be due to the relatively viscous nature of the phospholipid-based aerosols. The more uniform spray pattern ensures uniform and optimum administration and eventually allows it to reach the lung.

Fig. 4 shows the relative amount of dose retained at various parts of the airway penetration testing

Table 1
Characteristics of the various pressurized packed systems containing rifampicin

Parameters	Drug solution	Neutral liposomes (PC:Chol)	Negatively charged liposomes (PC:Chol:DCP)	MBSA-coated liposomes (PC:Chol; MBSA)	*O*-SAP-coated liposomes (PC:Chol; *O*-SAP)
Appearance	Homogeneous	Homogeneous	Homogeneous	Homogeneous	Homogeneous
Leak test	Passed	Passed	Passed	Passed	Passed
Internal pressure (psig)	34.4 ± 1.6	33.2 ± 1.4	32.1 ± 1.8	32.5 ± 1.3	31.8 ± 1.7
Discharge rate (mg per actuation)	113.6 ± 4.8	101.9 ± 6.4	109.6 ± 5.7	107.2 ± 4.6	102.1 ± 6.2
Penetration efficiency (%)	46.5 ± 2.6	41.4 ± 3.2	40.6 ± 2.7	38.2 ± 1.8	38.7 ± 2.5
Spray area at 15 cm (cm^2)	15.2 ± 2.6	13.8 ± 1.8	13.4 ± 1.6	12.9 ± 2.1	12.6 ± 1.5

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

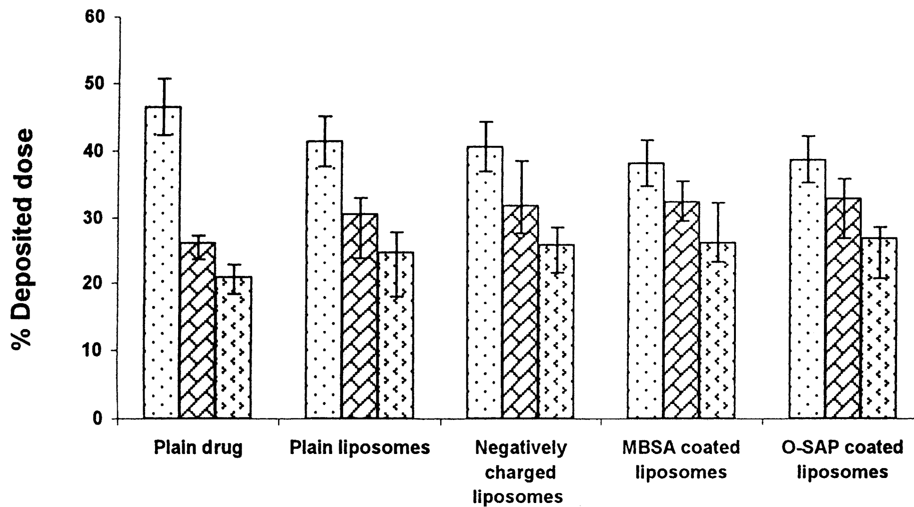

Fig. 4. Relative retention of deposited dose in various regions of the airways penetration efficiency testing apparatus from various formulated aerosolized systems. It was expressed as the percentage of the dose that eventually deposited on filter (▫), bronchi (▨), and trachea (▫). Data points are mean ± S.D. ($n = 6$).

apparatus. The observations are also recorded in Table 1. When plain drug solution aerosol was administered, a marginally increased amount of drug was measured on filter compared to liposomal aerosols. This could be due to minimum retention of plain drug in upper airways. Unlike plain drug, in case of phospholipid aerosol inhalation 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, which may to be due to relatively slow evaporation of propellant in the presence of phospholipids. A marginal decrease in the mean vesicle size was found in the case of aerosolized liposomes when compared with the preformed liposomal formulations. The percent entrapped drug was also found to be marginally low in the case of aerosolized liposomes (Table 2). The decrease in the mean vesicle size and percentage retention of the liposomal aerosols suggests that liposomes and liposomal aggregates could have broken up during passage through the delivery nozzle (atomizer) of the aerosol pack, resulting in a marginal decrease in mean vesicle size and percent drug entrapment. Vesicular fragmentation is most likely to occur as liquid is drawn up into the liquid inlet tube and gets mixed up with high-speed air jet. However, the marginal changes observed are thought to be insignificant as the aerosol droplet size and not the liposome size or composition is a determinant for aerosol deposition.

The percent viability of *M. smegmatis* inside macrophages (in vitro) after administration of drug (in vivo) is shown in Table 3. The time interval between drug treatment in vivo and isolation of macrophages

Table 2
Vesicle size and entrapment efficiency of rifampicin-loaded aerosolized and preformed liposomal systems

Liposome composition	Preformed liposomal systems		Aerosolized pressure packed liposomes	
	Vesicle size (μm)	% Entrapment	Vesicle size (μm)	% Entrapment
PC:Chol (7:3)	2.32 ± 0.48	47.4 ± 2.7	1.93 ± 0.25	42.3 ± 2.5
PC:Chol:DCP (7:3:0.1)	2.50 ± 0.54	49.8 ± 3.1	2.15 ± 0.32	43.5 ± 3.4
PC:Chol (7:3); MBSA	3.64 ± 0.65	46.5 ± 2.5	3.17 ± 0.47	41.1 ± 2.8
PC:Chol (7:3); *O*-SAP	3.85 ± 0.59	45.7 ± 3.4	3.24 ± 0.58	40.4 ± 2.3

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

Table 3
Percent viability of *Mycobacterium smegmatis* within the macrophages (in vitro) after the administration of the formulations in vivo

Macrophage-specific system	% Viability
Original smear viability of *M. smegmatis*	76.0 ± 5.6
Normal macrophages + *M. smegmatis*	69.5 ± 5.2
Free drug + normal macrophage + *M. smegmatis*	45.7 ± 4.7
Plain neutral liposomes + normal macrophage + *M. smegmatis*	31.6 ± 3.8
Negatively charged liposomes + normal macrophage + *M. smegmatis*	21.6 ± 3.1
MBSA-coated liposomes + normal macrophage + *M. smegmatis*	10.9 ± 2.1
O-SAP-coated liposomes + normal macrophage + *M. smegmatis*	7.1 ± 1.6

Wistar albino rats were administered aerosolized drug solution, drug bearing liposomal formulations. The macrophages were isolated and the macrophage monolayers were exposed to *M. smegmatis* (2×10^6 CFU). The interaction of drug-loaded macrophages and the bacilli was examined and the percent viability inside the macrophages was calculated by the FDA/EB staining procedure, and by counting acid-fast stainable bacteria by Ziel Neelson's technique. Results are shown as mean ± S.D. ($n = 6$).

for challenging *M. smegmatis* in vitro was selected on the basis of experiments performed at various time intervals. However, as the best results were seen at 48-h time interval, the experiments were designed accordingly. The original tissue smear viability was found to be 76.0 ± 5.6%. The viability of *M. smegmatis* in control macrophages was 69.5±5.2%. The percent viability reduced to 45.7 ± 4.7% in case of drug-treated macrophages and values were further declined on liposome encapsulated drug treatment/administration (31.6 ± 3.8%). However, exceptional low percent viability was recorded with ligand-appended liposomal formulations, which was in the range of 7–11%. The difference in efficacy of *O*-SAP-coated liposomes over the MBSA-coated liposomes could be attributed to the preferential accumulation in the alveolar macrophages, as reflected in in vivo distribution studies. The results are superior to those observed when the drug was administered either as a plain solution or encapsulated in plain liposomes. Further, they indicate the ability of ligand-appended liposomes to inactivate macrophage localized/harbored *M. smegmatis*. Ideally, 0% viability could not be achieved in any case due to one of the several reasons. It may not be possible that all macrophages had sequestered and taken up the liposomes after administration. Therefore, some macrophagic tropics may remain untreated where mycobacterium could survive and proliferate. However, on repeated subsequent dosing the ideal situation of 0% viability may be achieved.

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 rifampicin after administration of different liposomal formulations and plain drug solution. All the developed liposomal formulations (both ligand appended and negatively charged) showed greater accumulation in the lungs when compared with the controls. In the case of free drug solution, only 39.12 ± 3.7% of the administered dose was found in the lungs at 0.5-h post-administration and almost an equal amount was recovered in serum (29.84 ± 2.3%). 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. Plain neutral liposomes (based on PC:Chol) showed a initial higher lung accumulation (49.03 ± 4.6% after 0.5 h) but the overall distribution pattern with time was comparable with that of free drug and negligible to nil drug concentration was found in lungs after 24 h. They readily distributed to systemic circulation from where they were rapidly taken up by the organs of reticuloendothelial system (RES), mainly liver and spleen, and cleared (digested) by the fixed tissue macrophages there in (Kirsh et al., 1987; Scherphof, 1991). In the case of negatively charged liposomes (PC:Chol:DCP), 53.86±5.4% drug accumulation was recorded in lungs after 0.5 h and only 19.72 ± 1.3% drug was found in serum. At the end of 6 h, significantly higher concentration of the drug (29.67 ± 2.9%) was estimated in lung tissues and 4.14 ± 0.6% drug concentration was estimated even after 24 h. This may be due to selective intervention and capture of negatively (anionic) charged liposomes via scavenger receptors expressed

Table 4
Organ distribution of rifampicin following administration of various aerosolized formulations

Formulations	Organ	% Dose recovered after					
		30 min	1 h	2 h	4 h	6 h	24 h
Free rifampicin							
	Serum	29.84 ± 2.3	35.76 ± 2.8	40.94 ± 3.6	31.05 ± 2.5	18.12 ± 1.2	5.39 ± 0.92
	Liver	6.74 ± 1.3	11.23 ± 1.5	14.63 ± 1.7	10.72 ± 1.4	4.45 ± 0.73	2.18 ± 0.12
	Spleen	1.42 ± 0.11	2.01 ± 0.15	2.46 ± 0.32	2.89 ± 0.46	0.93 ± 0.04	ND
	Lung	39.12 ± 3.7	27.14 ± 2.3	19.78 ± 1.6	14.55 ± 1.2	8.73 ± 1.4	ND
	Kidney	ND	1.12 ± 0.11	2.15 ± 0.26	3.76 ± 0.84	6.27 ± 0.42	0.73 ± 0.06
Plain neutral liposomes (PC:Chol)							
	Serum	23.67 ± 1.4	26.94 ± 1.9	29.18 ± 2.6	22.09 ± 2.1	15.97 ± 1.7	5.32 ± 1.1
	Liver	5.32 ± 0.82	13.89 ± 1.7	24.04 ± 2.7	28.54 ± 1.8	18.76 ± 1.1	2.76 ± 0.25
	Spleen	1.13 ± 0.08	1.97 ± 0.11	2.12 ± 0.17	2.62 ± 0.23	1.93 ± 0.18	ND
	Lung	49.03 ± 4.6	35.76 ± 3.8	22.18 ± 3.2	18.17 ± 2.7	11.36 ± 2.1	ND
	Kidney	ND	1.43 ± 0.12	2.58 ± 0.22	4.81 ± 0.72	7.31 ± 1.1	0.81 ± 0.04
Negatively charged plain liposomes (PC:Chol:DCP)							
	Serum	19.72 ± 1.3	22.47 ± 1.6	27.13 ± 2.4	21.43 ± 1.8	15.32 ± 1.4	4.21 ± 0.75
	Liver	4.83 ± 0.62	7.56 ± 1.1	9.37 ± 1.7	15.86 ± 1.9	12.74 ± 1.6	2.15 ± 0.18
	Spleen	0.97 ± 0.14	1.21 ± 0.16	1.85 ± 0.17	2.42 ± 0.20	1.69 ± 0.08	ND
	Lung	53.86 ± 5.4	45.79 ± 4.3	39.93 ± 3.7	35.17 ± 3.1	29.67 ± 2.9	4.14 ± 0.60
	Kidney	ND	0.98 ± 0.09	1.72 ± 0.23	2.72 ± 0.31	4.62 ± 1.1	0.94 ± 0.06
MBSA-coated liposomes (PC:Chol; MBSA)							
	Serum	11.31 ± 1.1	16.28 ± 1.6	20.77 ± 2.3	17.35 ± 1.9	14.98 ± 1.2	3.12 ± 0.25
	Liver	3.12 ± 1.2	5.72 ± 2.3	8.14 ± 1.7	12.67 ± 1.5	9.34 ± 1.1	2.08 ± 0.12
	Spleen	0.68 ± 1.1	1.05 ± 0.05	1.73 ± 0.14	2.27 ± 0.06	1.93 ± 0.01	ND
	Lung	61.49 ± 6.3	54.04 ± 5.2	48.16 ± 4.5	43.91 ± 4.1	39.57 ± 3.3	8.12 ± 1.6
	Kidney	ND	0.87 ± 0.04	1.30 ± 0.07	2.81 ± 0.12	4.32 ± 0.74	0.94 ± 0.02
O-SAP-coated liposomes (PC:Chol; *O*-SAP)							
	Serum	9.21 ± 1.2	15.87 ± 1.6	19.65 ± 2.2	17.11 ± 1.8	15.27 ± 1.2	3.07 ± 0.73
	Liver	3.05 ± 1.1	5.93 ± 1.4	8.91 ± 2.2	13.66 ± 1.3	10.23 ± 1.2	2.12 ± 0.18
	Spleen	ND	0.78 ± 0.03	1.62 ± 0.11	2.14 ± 0.09	1.17 ± 0.03	ND
	Lung	65.14 ± 7.8	56.37 ± 6.8	50.74 ± 6.3	45.18 ± 5.4	41.55 ± 4.2	10.75 ± 1.8
	Kidney	ND	0.42 ± 0.01	0.98 ± 0.03	1.81 ± 0.06	2.73 ± 0.11	1.06 ± 0.21

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

on alveolar macrophages (Fidler et al., 1980). The lung uptake of the ligand-appended liposomes was higher compared to the negatively charged and neutral liposomes. After administration, the MBSA-coated liposomal aerosol was selectively accumulated with their contents into the lungs. The lung accumulation levels estimated were 61.49 ± 6.3%, 39.57 ± 3.3%, and 8.12 ± 1.6% of the administered dose after 0.5, 6, and 24 h, respectively, after administration. The high affinity of MBSA as an anionic ligand specific for type I and type II scavenger receptors of mammalian macrophages could be accounted for higher levels of accumulation (Rigotti et al., 1995). The developed system is a ligand-anchored system selective for the delivery of rifampicin to macrophages because these receptors are exclusively expressed in abundance on the surface of mature macrophages at later stages of differentiation. Moreover, these receptors have no apparent ligand-induced downregulation of receptors and have been shown to be effective in rapid clearance of the ligand-bound drug from the extracellular medium to phagolysosome of macrophages.

Liposomes coated with *O*-SAP showed 65.14 ± 7.8% and 41.55 ± 4.2% accumulation in lungs at 0.5- and 6-h post-administration. Significantly higher concentration of the drug (10.75 ± 1.8%) was retained in the lung even after 24 h. The observed higher accumulation of these liposomes in alveolar macrophages

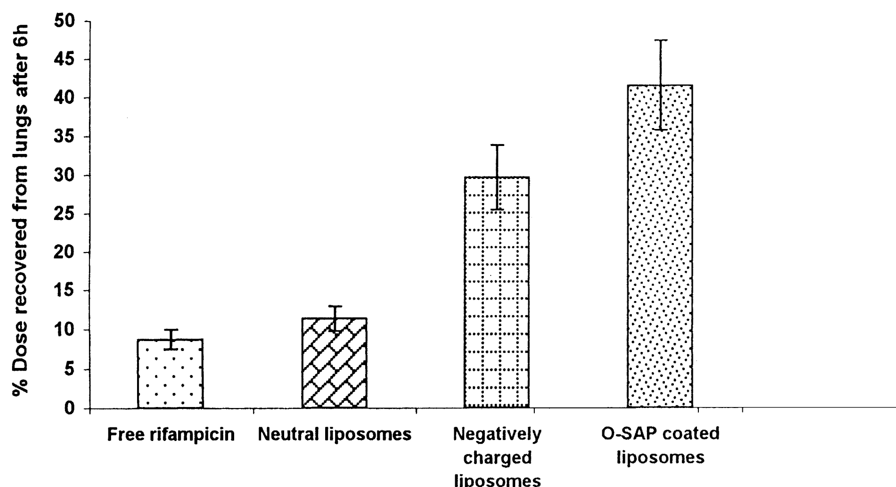

Fig. 5. Relative lung retention of various formulations after 6-h administration.

may be due to their affinity for amylopectin-coated liposomes (Takada et al., 1984). Alveolar macrophages mainly contribute to the lung uptake of *O*-SAP-coated liposomes. This has been confirmed by the fact that a fluorescent probe encapsulated in such liposomes is found substantially localized in the alveolar macrophages and other phagocytes after intravenous injection (Sunamoto et al., 1987).

The lung accumulation of *O*-SAP-coated liposomes at 0.5 h after intravenous injection was found to be nearly 27% (Takada et al., 1984) while only 3.8% of administered dose was recovered after 24 h. Deol and Khullar (1997) reported nearly 19% lung accumulation of *O*-SAP-coated liposomes at 1 h after intravenous injection. However, in the present study aerosolization of *O*-SAP-coated liposomes resulted in lung accumulation of $56.37 \pm 6.8\%$ of administered dose after 1 h. The present study reveals that aerosolization of ligand-appended liposomes containing antibacterial provides a possible means of drug targeting to alveolar macrophages. The technique circumvents and addresses the limitations, like possible leakage of the contents en route 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.

Fig. 5 shows the comparative percent drug recovered from lungs after 6-h administration of various formulations. The values are 1.3 times for plain neutral liposomes (PC:Chol), 3.4 times for negatively charged liposomes (PC:Chol:DCP), 4.53 times for MBSA-coated liposomes (PC:Chol; MBSA), and 4.76 times for *O*-SAP-coated liposomes (PC:Chol; *O*-SAP) 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 concentrations 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.

4. Conclusion

Results of this study suggest that encapsulation of anti-tubercular drugs in the liposomes, modification of the liposomal surface by incorporating negative charge, and using macrophage-specific ligands and deposition to respiratory tract via aerosolization will certainly improve the chemotherapy against pulmonary tuberculosis. 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 *M. tuberculosis* infection.

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