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Ligand directed macrophage targeting of amphotericin B loaded liposomes

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Abstract

Two types of ligand anchored multilamellar liposomes (MLVs) containing amphotericin B (Amp B) were prepared. The MLVs consisting of soya phosphatidylcholine (PC) and cholesterol (Chol) were coated with *O*-palmitoyl mannan (OPM). Similarly, the MLVs with the same Amp B content consisting of soya PC, Chol and phosphatidylethanolamine (PE) were prepared and covalently anchored with *p*-aminophenyl-mannopyranoside (PAM). The surface modified MLVs and their plain counterparts were characterised for size, shape, lamellarity, entrapment efficiency and ligand density. The stability in serum and in vivo bio-distribution in albino rats were also determined. It was observed that extent of accumulation of liposomal Amp B in macrophage rich organs, particularly liver, spleen and lungs was significantly high when compared against the free drug. The rates and extent of accumulation were found to increase further on ligand anchoring. In either of the cases, the macrophagic uptake of ligand anchored liposomes was inhibited significantly on pre-injection of hydrolysed mannan, being suggestive of receptor mediated uptake of ligand anchored liposomes. Comparison of biodistruibution pattern of ligand anchored MLVs revealed that PAM linked liposomes exhibited a higher hepato-splenic accumulation where as drug accumulation in lungs was highest in the case of OPM coated liposomes. It was thus observed that mannopyranoside is a specific ligand for targeting bioactives to the macrophages of liver and spleen while OPM could preferentially negotiate the targeting of bioactives to the alveolar macrophages.

Keywords: Liposomes; Amphotericin B; *O*-palmitoyl mannan (OPM); *p*-Aminophenyl-mannopyranoside (PAM); Macrophage targeting

1. Introduction

Targeted drug delivery to the macrophages has been appreciated as a strategy for achieving diverse objectives like treatment of lysosomal storage diseases (Gordon and Rabinowitz, 1989), targeting of immunomodulators to activate macrophages (Fidler, 1988), cell or cell product depletion (van Rooijen and Sanders, 1994) and blockade of the macrophages (O'Mullane et al., 1987). Many approaches for targeting the drugs to the macrophages have been developed, which are largely represented by liposomes (Alving,

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1982; Bakker-Wouderberg et al., 1994). Although liposomes show natural affinity towards the macrophages and passively targeted to them; yet inclusion of the macrophage receptor(s) specific ligands may significantly enhance the rates and extent of liposomal uptake by the macrophages.

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 localisation 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 rationale approach to the problem requires that drugs should be targeted to the macrophages in such a way that the interaction of the free drug with non-target tissues could be minimised. 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). Toxicological comparison of free and liposomally formulated Amp B in mice has revealed that maximum tolerated dose has been significantly increased. The LD_{50} has been found to increase from 1.2 to more than 12 mg kg^{-1} in the case of mice (Lopez-Berestein et al., 1983). Although liposomal formulations of Amp B could considerably reduce the toxicity of the drug and subsequently make it possible to enhance the therapeutic index, however, their pharmacokinetic and pharmacodynamic profile still needs to be improved.

Amp B bearing liposomes investigated so far have been small unilamellar or MLVs. The marketed liposomal formulation, AmBisome™ (Nexstar, Boulder, CO, USA), similar to other marketed lipid formulations of Amp B like ABELECT™ (Amp B-lipid complex, The Liposome Company, Princeton, NJ, USA) and AMPHOTEC™ (Amp B colloidal dispersion, Sequus Pharmaceuticals, Merlo, CA, USA), preferentially accumulate in the liver and spleen of animals (Hiemenz and Walsh, 1996). However, the rate of uptake of AmBisome by the reticuloendothelial system appears to be much slower than by ABELECT or AMPHOTEC. It is hypothesised that the larger lipid complexes and dispersions may be readily phagocytised by the macrophages of the reticuloendothelial system than the small unilamellar vesicles of AmBisome. However, this may account for the higher peak plasma levels and prolonged circulation times compared with its larger counterparts. This creates a discrepancy that whether the chemotherapeutic effect of Amp B is due to the localisation of the drug inside the intracellular pathogen infected macrophages or to the slow and sustained release of free Amp B in the circulation and tissues.

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 hepatosplenic 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 utilised for targeting bioactives to the macrophages (reviewed in Vyas, 2000). The receptor facilitates endocytosis of glycoproteins terminated with mannose, fucose and glucosamine. Mannose residues have been appended to liposomes as mannosylated albumin (Garcon et al., 1988); palmitoylated polysaccharides (Sunamoto et al., 1985); mannobiose arachidonic esters (Yachi et al., 1995); mannose terminated glycolipids (Barrat et al., 1986); mannopyranoside (Bachhawat et al., 1984) and mannose terminated glycoproteins (Szoka and Mayhew, 1983; Ponpipon et al., 1984) for macrophage targeting.

The present work was programmed for designing an actively targeted system of Amp B based on liposomes. The macrophages being the target were assessed for selective accessibility through receptor-mediated endocytosis using *O*-palmitoylated mannan (OPM) and *p*-aminophenyl-mannopyranoside (PAM) as specific ligand modules. Comparative in vivo distributions and targeting profiles of OPM and PAM anchored liposomes against plain liposomes were studied.

2. Materials and methods

2.1. Materials

Amp B was obtained as a gift sample from M/S Ambalal Sarabhai Enterprises, Vadodara, India. Soya PC, cholesterol (Chol), phosphatidylethanolamine (PE), PAM, stearylamine, mannan and concanavalin A were purchased from Sigma (USA). Palmitoyl chloride (Fluka, Switzerland), glutaraldehyde (Loba-Chemie, India), absolute alcohol (Bengal Chemicals, India) were purchased and used as supplied. All other chemicals were of analytical grade until and used as procured.

2.2. Preparation of OPM coated MLVs containing Amp B

2.2.1. Preparation and optimisation of MLVs containing amphotericin B

Multilamellar vesicles (MLVs) containing Amp B were prepared by the method described by Lopez-Berestein et al. (1983). Soya PC and Chol were dissolved in the minimum amount of chloroform and a methanolic solution (60 μg ml^{-1}) of Amp B was added to it. Soya 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% moles 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°C for 60 min and subsequently at room temperature for 6 h. The dispersion was centrifuged at 60 000 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 (0.5% w/v), drug content was determined spectrophotometrically at 404 nm and percent drug entrapment was calculated. MLVs with optimum Amp B to lipid ratio were optimised for optimum Soya PC to Chol ratio in terms of percent drug entrapment and toxicity towards erythrocytes. The MLVs with different Soya PC to Chol ratios (90:10, 80:20, 70:30, 60:40, 50:50 molar ratios) were prepared. Amp B content however was kept constant at its optimum concentration level. The liposomes were evaluated for percent entrapment and toxicity to mammalian cells in terms of percent hemolysis. Percent hemolysis was determined by the method described by Mehta et al. (1984). Liposomal dispersions containing equivalent amount of Amp B (50 μg ml^{-1}) were incubated with 1.0 ml mammalian blood at 37 ± 1°C for 45 min, centrifuged at 60 000 rpm for 4 h 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% hemolysis). From the haemoglobin released in supernatant, percent hemolysis in each case was computed and assessed. Percent hemolysis and percent entrapment were plotted against Soya PC to Chol ratio, from which optimum Soya PC to Chol ratio was determined.

2.2.2. Synthesis, characterisation and incorporation of O-palmitoyl mannan (OPM) into preformed MLVs containing Amp B

O-palmitoylated mannan was synthesised from the yeast mannan by the process reported elsewhere in the literature (Hammerling and Westphal, 1967). *O*-palmitoyl mannan, in brief, was prepared by reacting mannan (1.0 g) in dry 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 ± 1°C for 1 h.

Hydrophobised mannan (OPM) was characterised by infra red (IR) spectroscopy to identify carbonyl groups, and thus, to ascertain that mannan is covalently bound to palmitoyl anchor. The IR spectrum of OPM and mannan (1%), incorporated into a KBr disc, was run on a FT-IR single beam spectrometer. Characteristic peaks were recorded at 2650, 1480, 1690, 1210–1190, and 3400 cm^{-1}.

Coating of the Amp B loaded MLVs with hydrophobised derivative of mannan was affected by incubating 1.0 ml of plain liposomal suspension with the dispersion of optimised amount of OPM (0.15:1 w/w OPM:PC) 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 resulting suspension through a sephadex G-50 mini-column at 2000 rpm for 5 min. For optimisation of OPM: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. 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 micromhos. Specific electrical conductance was plotted against the OPM:PC ratio and optimum OPM:PC ratio was determined from the plot as one, at or beyond which no further changes in the conductance 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 specific electrical conductance of these vesicles was recorded after separating the excessive, unbound polysaccharide. From the plot of Specific electric 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. Preparation of mannopyranoside (PAM) linked MLVs containing Amp B

2.3.1. Preparation of PE based liposomes

MLVs containing optimum amount of Amp B (from the earlier experiment) and different molar ratios of PC, PE and Chol were prepared by the method discussed. MLVs obtained were evaluated for the shape, size, percent drug entrapment and toxicity to mammalian cells in terms of percent hemolysis as suggested in the earlier experiments. From these parameters optimum proportions of the phospholipids were determined and selected.

2.3.2. Linkage of mannopyranoside (PAM) to MLVs

Mannosylated ligand (PAM) was inserted in to the lipid bilayer using the PE end groups. *p*-Aminophenyl-mannopyranoside was linked to PE containing MLVs by the method described by Ghosh et al. (1982). A 1.0 ml liposomal dispersion (containing $\cong 30$ mg lipids per ml) in 0.9% w/v aqueous NaCl solution was mixed with 20.0 mg PAM contained in 2.0 ml of aqueous NaCl solution. Glutaraldehyde was then slowly added to the suspension to a concentration level 3 mM and the mixture was incubated for 5 min at 20°C. Uncoupled glycosides and glutaraldehyde were removed by dynamic dialysis technique against 0.9% NaCl solution.

2.4. Characterisation of liposomes coated with mannose terminating ligands

Developed formulations were characterised prior to and after surface ligand anchoring. The different liposomal formulations were evaluated for their shape and vesicle type by transmission electron microscopy (TEM). Vesicle characterisation for size and shape was performed using TEM. Phosphotungstic acid (1%) was used as a negative stain (JEM 1200, EX 11, JEOL, Japan). Carbon coated samples were placed over a copper grid and subjected to TEM analysis. Vesicle size distribution was also assessed using a phase contrast

microscope (Leitz, Biomed, Germany). Vesicle dispersions were appropriately diluted and wet mounted on a haemocytometer and photographed using the microscope. The negatives were projected on a piece of calibrated paper using an enlarger with an adjusted magnification (× 1250). Diameters of around 500 vesicles were noted for each system and average vesicle size was calculated.

The surface charge of the vesicles was calculated using zeta potentiometer (Aplex 35, France). The mobility of various liposomes in PBS (pH 7.4, 0.01 M) was determined using Helmholtz–Smoluchowski's equation (Adamson, 1967). The formulations which were free from undesired and unspecified liposomal structures were evaluated for the entrapment efficiency by the method described by New (1990). The percent entrapment was determined and expressed as the percentage of added drug incorporated in the vesicles. The yield was reported as the ratio of experimentally measured amount of the drug in the dispersion and the actual amount used for the entrapment. Drug content of the MLVs was determined by the method given by Lopez-Berestein et al. (1983), following the disruption of liposomal pellets by adding triton X 100 (0.5% w/v). The presence of mannose residues on the surface of liposomes was detected by agglutination of the vesicles with concanavalin A. In the case of mannopyranoside linked liposomes, the percent of total PE that gets modified with the mannopyranoside linkage was determined by titrating amino groups of liposomal PE with trinitrobenzene sulphonic acid in the presence of 0.5% triton X-100.

2.5. Stability in serum

The stability of liposomes in the serum was determined by observing vesicle disruption and intact vesicles count and drug leaching following incubation of MLVs with serum at 37 ± 1°C. 1.0 ml suspension of the MLVs was incubated with 2.0 ml serum at 4 ± 1 and 37 ± 1°C for 2 h and the MLVs counts per cubic mm of the dispersion were recorded by the microscopic method using haemocytometer and nauebaer's chamber (Japan). Percent vesicles remaining in dispersion as intact was calculated. The drug content of the MLVs was determined by the method given by Lopez-Berestein et al. (1983). Each formulation (1.0 ml) was incubated with 2 ml serum at 37 ± 1°C for 1, 2, 4, 6 and 24 h. After specified time intervals, suspensions were centrifuged at 60 000 rpm for 4 h and supernatant was filtered through 0.45 μm membrane filter. The filtrate was analysed for drug content by reverse phase high performance liquid chromatography (HPLC) method as described elsewhere (Van Etten et al., 1993).

2.6. Determination of in vivo target specificity

Albino rats of either sex weighing about 150–200 g each were divided in seven groups of 18 rats each. Food and water were allowed ab libitum during the study period. Free Amp B (0.8 mg kg^{-1} body weight), ligand appended formulations and their respective non-ligand anchored counterparts containing equivalent doses of Amp B (0.8 mg kg^{-1} body weight) were administered intravenously to different groups. In order to observe the effect of hydrolysed mannan on the uptake of ligand appended liposomes, two groups were administered with 10.0 mg hydrolysed mannan i.v. prior to the administration of OPM coated and PAM anchored formulations. Three albino rats from each group were sequentially sacrificed at 15, 30 min, 1, 2, 4, and 24 h after administration of the formulations. Blood was collected by cardiac puncture method. Different organs (liver, spleen, lungs, kidney) were excised, isolated, washed with distilled water and were blot dried using tissue papers (Van Etten et al., 1995). Drug content in the blood and organs was determined by HPLC method (Van Etten et al., 1993).

Drug localisation index for each organ was calculated using the data from biodistribution studies by the formula given by Gupta and Huang (1989).

$$\text{Drug localization index} = \frac{\text{Drug concentration in target tissue at time '}t\text{' after administration of test delivery system}}{\text{Drug concentration in target tissue at time '}t\text{' after administration of free drug}}$$

3. Results and discussions

Two different liposomal formulations, one based on phosphatidylcholine and the other based on phosphatidylcholine and PE as the constitutive lipids, were prepared and anchored with macrophage specific ligands, OPM and PAM, respectively. The drug, Amp B was incorporated in to the liposomes using lipid film hydration technique.

Liposomes prepared from PC as a constitutive lipid were optimised for various parameters. These include the molar ratio of Amp B to total lipid, ratio of Chol to total lipid at an optimised Amp B concentration, and also the coating ratio and incubation time of the OPM.

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 associations of Amp B molecule with Chol may result in leaky membrane formations 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 be free of other undesired structures and average vesicle size measured was 2.37 ± 0.76 μm.

In another variation, with an increase in Chol concentration in liposomes at constant and optimised 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 10:90 to 40:60 the percent hemolysis was recorded between 11.2 ± 1.09 and 1.0 ± 0.19% (Fig. 1). The mechanism responsible for toxicity reduction of Amp B upon liposomal entrapment is still undefined. However, it seems 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. 1). It may probably be due to sterically favourable and hence, preferential accommodation of the Chol molecules with in the bilayer assemblages. Optimum PC:Chol molar ratio was found to be 7:3, which could entrap maximum amount of drug (79.2 ± 1.29) with acceptably lower levels of toxicity towards erythrocytes (percent hemolysis = 1.1 ± 0.18).

After optimising the process parameters, PC liposomes were coated with OPM, which was characterised by IR analysis. Comparison of the infra red spectra of OPM with mannan revealed the presence of extra peaks due to C–C band deformation (2850 cm^{-1}) and C–H deformations (1480 cm^{-1}) arising from alkyl group in the product. Peak that corresponds to C=O stretching vibrations expected at 1735 cm^{-1} (Pavia et al., 1979) appeared with a shift at 1695 cm^{-1}. It may be a consequence of intra-molecular hydrogen bond formation be-

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 ($n = 3$)
20	Comma shaped ribbons and other unspecified structures	Not determined
16	Unspecified structures, ribbons and some liposomal population	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 1.86 μm	68.9 ± 1.23%
2	Only liposomes of size range lower than 1.6–6.4 μm with average size 2.37 μm	79.2 ± 1.56%
1	Liposomes of size range 1.6–4.8 μm with average size 3.86 μm	84.1 ± 1.33%

[a] Percent molar ratio of Amp B to total lipids.

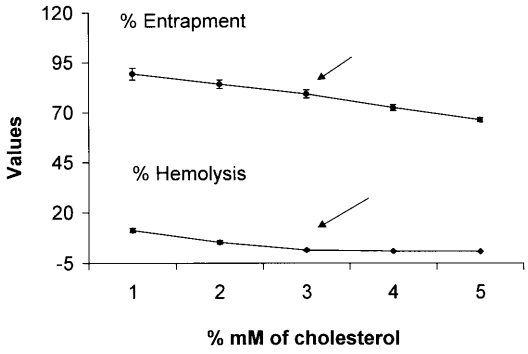


Fig. 1. Optimisation of Chol content in MLVs containing Amp B in terms of percent hemolysis (◆) and percent entrapment (●). Percent hemolysis was determined by incubation of freshly pooled rat erythrocytes with liposomes (PC:Chol) containing different Chol and 2% M of Amp B to total lipids. The arrow has denoted optimised value. Percent entrapment was determined and 30 mM Chol was found to provide sufficiently high values with considerably low toxicity.

tween carbonyl and hydroxyl groups which suppress the stretching force constant of C=O bond. The presence of hydrogen bonding was further confirmed by the lower frequency stretching vibrations of the O–H band (at about 3388 cm^{-1}) and also by its higher intensity and larger bandwidth. C–O stretching vibrations appeared as a characteristic band in the range 1210–1190 cm^{-1}. All these peaks in the infra red spectra provided convincing evidences of the formation of an ester bond between mannan and *O*-palmitoyl anchor.

The OPM:PC ratio and incubation time were optimised 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 3306.4 ± 12.1 to 2348.2 ± 11.2 μmhos, when the OPM:PC ratio was increased from 0.01:1 to 0.15:1 (Fig. 2a). 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, while optimum incubation time (the time that corresponds to completion of OPM coating under the experimental condition) recorded was 1.5 h (Fig. 2b). At the optimum OPM concentration level, the optimum incubation time was recorded to be 1 after which electrical conductance remained fairly constant at 2388 ± 12.7 μmhos.

The other liposomal formulation was prepared from PC and PE as constitutive lipids and optimised for parameters including PC to PE ratio and Chol to total lipid ratio. The optimisation was carried out to get an optimum vesicle size coupled with a higher entrapment and an acceptable toxicity towards erythrocytes. PE containing liposomes were evaluated and optimised for total PE content. It was found that additional PE compensated with an equivalent reduction in PC exhibited no significant effect on the toxicity of Amp B towards erythrocytes as well as on percent entrapment so far the concentration of Chol was kept

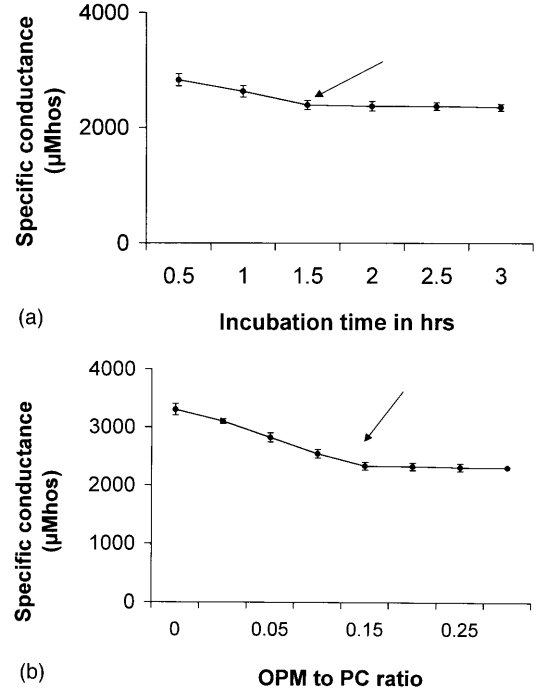
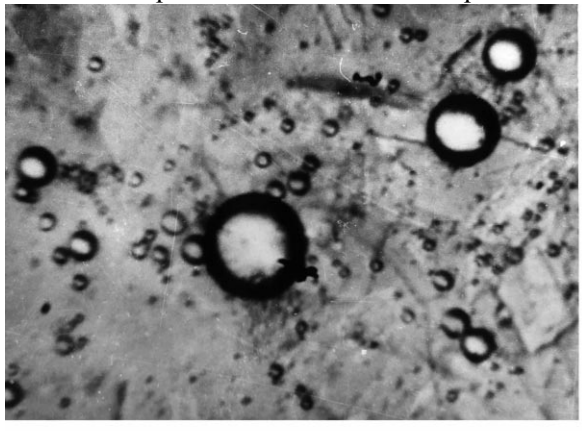


Fig. 2. Optimisation of OPM:PC ratio and incubation time in terms of specific conductance at an applied voltage of 10 mV for the completion of coating process. For optimum OPM:PC ratio formulations were incubated for 4 h with different amount of OPM (A). For optimising incubation time liposomes with an optimum OPM:PC (0.15:1) were incubated for different time intervals (B). Completion of coating was indicated by no further changes in specific conductance (denoted by arrow).

constant (Table 2). However, when PE:PC ratio raised near 1:1 molar ratio, the vesicles were off spherical in shape and distorted vesicles were seen. This can be attributed to the reported instability of the PE because of its rapid phase transition to non-bilayer (hexagonal phase II) structures (Litzinger and Huang, 1992). However, when the PE:PC ratio was decreased relatively stable vesicles were obtained, owing to increased stabilising contribution of PC and Chol and hence less membrane defects. Optimum PC:PE:Chol ratio was found to be 5:2:3 mM. Liposomes formed at this composition were absolutely spherical within a size range 1.66 to 4.98 μm. 87 ± 2.5% of liposomal population was lower than 3.50 μm in size. The average size of the vesicles was 1.78 ± 0.35 μm, which increased marginally on anchoring PAM residues via glutaraldehyde spacer arm (Table 3).

The optimised formulations used for further in vitro and in vivo studies were coded as PC3 (PC liposomes-plain), OPMPC3 (OPM coated), CE3 (PC/PE liposomes-plain), and PAMCE3 (PAM coated CE3). Ligand appended liposomes were characterised for vesicle shape, size and size distribu-



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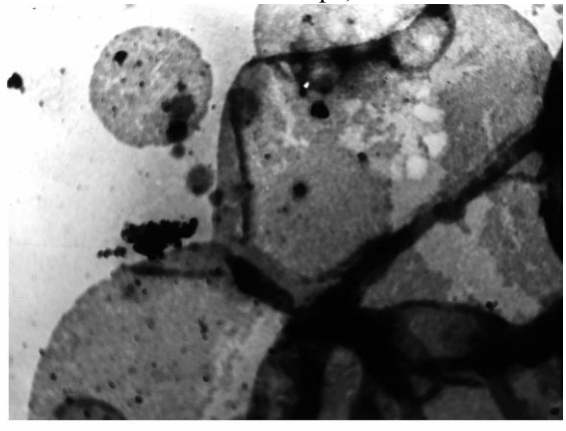

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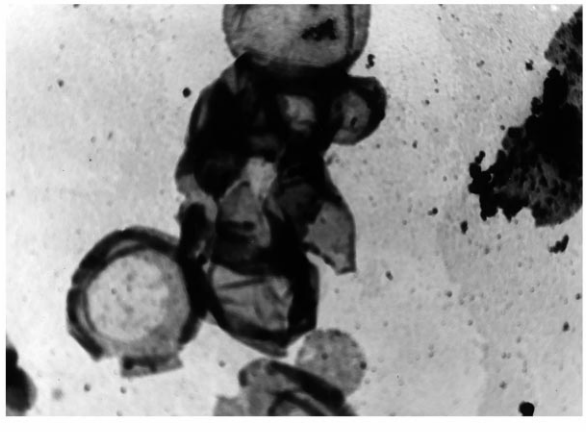

3

Photomicrographs show the surface coating/anchoring of ligands on the amphotericin B loaded liposomes.

1. Coating is signified with extinguished multilamellarity and opaque appearance.
2. Surface anchoring and inter-digitization of mannose-terminating ligand (PAM).
3. Coating of hydrophobized derivative of mannan (OPM).

Table 2
Optimisation of PC:PE:Chol ratio

Molar ratios PC:PE:Chol	Entrapment (%, $n=3$)[a]	Hemolysis (%, $n=3$)[a]
7.0:0.0:3.0	79.2 ± 1.12	1.5 ± 0.021
6.0:3.0:1.0	81.8 ± 1.24	13.1 ± 0.85
6.0:2.0:2.0	78.9 ± 1.18	6.9 ± 0.54
5.0:2.0:3.0	74.2 ± 1.21	1.6 ± 0.036
4.0:3.0:3.0	70.0 ± 1.24	1.8 ± 0.054
4.0:4.0:2.0	69.8 ± 1.54	7.3 ± 0.63

[a] All the values are representatives of mean ± S.D. for three independent determinations.

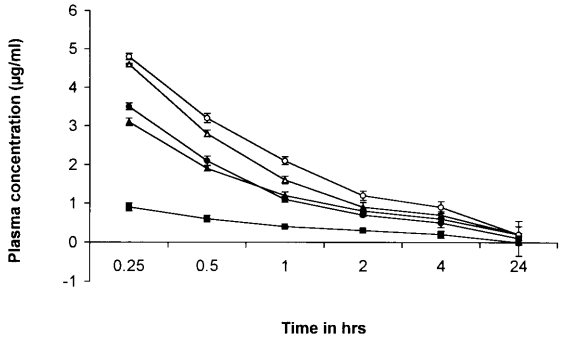

Fig. 3. In vitro drug leaching in serum from MLVs. MLVs was incubated with serum at 37 ± 1°C for different time intervals and drug concentrations in the serum were recorded. OPM coated formulation (▲) displayed the least drug leaching, followed by PAM linked liposomes (●). Among plain versions PC3 (△) and CE3 (○), the later exhibited greater drug leaching. Ligand anchoring was found to decrease drug leaching.

tion and percent entrapment and results are summarised in Table 3. The presence of coating on the liposomal surface could be appreciated from the TEM, which indicates surface interven-

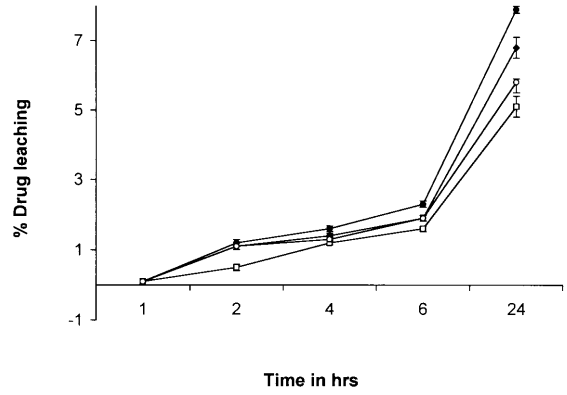

Fig. 4. Blood concentrations of Amp B following the intravenous administration of free Amp B (◆), PC3 (○), CE3 (△), OPMPC3(●) and PAMCE3 (▲). Blood concentrations in the case of liposomal preparations were considerably higher as compared with the free drug. Blood concentrations of the ligand-anchored formulations were lower than their respective plain counterparts.

ing and anchoring of mannose terminating ligands (photomicrographs 1–3).

The coating of OPM and PAM on the surface of liposomes was confirmed qualitatively by the agglutination of liposomes induced by concanavalin A (Iwamoto et al., 1991). The results suggest that surface ligand anchoring and the process used did not affect the ligand affinity and avidity towards their recognition motifs. The titration of liposomal PE groups with trinitrobenzene sulphonic acid revealed that about 15–18% of the total amino groups were modified via covalent coupling of PAM (data not shown). This finding further suggests that only surfacial PE be involved in the covalent ligand anchoring.

Table 3
Formulation codes, composition and characterisation of various ligand anchored and plain formulations

Formulation codes	Composition (mmole)	Nature of ligand	Percent encapsulation[a]	Average vesicle size[a]
PC3	PC:Chol (7:3)	–	79.6 ± 1.8	2.35 ± 0.25
OPMPC3	PC:Chol (7:3)	OPM	78.6 ± 2.1	3.04 ± 0.18
CE3	PC:PE:Chol (5:2:3)	–	74.2 ± 1.7	1.78 ± 0.35
PAMCE3	PC:PE:Chol (5:2:3)	PAM	73.9 ± 1.9	2.10 ± 0.06

[a] All the values are representatives of mean ± S.D. for three independent determinations.

Stability in serum was measured as percent vesicles remaining intact per mm^3 after the incubation for 2 h. The stability of liposomes (both plain and surface modified) was found to be adversely affected on incubation with freshly pooled rat serum. However, the liposomal preparations developed for targeting to macrophages should be cleared from the circulation within a very short span of time as reported elsewhere (Ghosh et al., 1982). Therefore, the observed instability in serum may not have discernible bearing on bio-disposition. The disrupted vesicles appearing as unspecified structures and aggregated liposomes were seen under microscopic observation. The loss of vesicles was not correlated well with the percent drug leached in serum even at 37 ± 1°C (Fig. 3). The percent of Amp B leached in the serum was lower (7.9 ± 0.71% in 24 h) than the expected values. This is presumably because Amp B being practically insoluble might have remained associated with disrupted bilayer fragments rather than diffusing in to the serum.

The biodistribution patterns studied clearly indicate the superiority of the liposomal Amp B as compared against the plain drug in increasing the accumulation of Amp B in the organs rich in macrophages (liver and spleen). The intravenous administration of free Amp B (0.8 mg kg^{-1} body weight) resulted in relatively lower plasma concentrations of Amp B (0.9 ± 0.12 μg ml^{-1}) after 15 min which further declined to 0.2 ± 0.1 μg ml^{-1} as estimated at 4 h and to negligible extent (not detectable) at 24 h. This could account for the rapid but non-specific tissue distribution of the free drug to various tissues of the body. This seems related to relatively low levels of drug recovered from macrophage rich organs like liver, spleen and lungs as compared with Amp B bearing liposomal formulations. Plasma concentrations of Amp B after the administrations of Amp B bearing plain liposomes were significantly higher than the free drug, i.e. 4.6 ± 0.7 and 4.9 ± 1.1 μg ml^{-1} after 15 min, respectively, in the case of formulations PC3 and CE3 (Fig. 4). Ligand anchored liposomes (OPMPC3 and PAMCE3) exhibited still higher plasma concentrations (3.1 ± 0.9 and 2.9 ± 1.1 μg ml^{-1}, respectively, after 15 min) than free drug, but it was noticeably lower than that obtained after the administration of their plain counterparts (PC3 and CE3).

This decrease in plasma concentration in the case of ligand anchored liposomes was accompanied by a corresponding increase in drug accumulation in macrophage rich organs like liver, spleen and lung. The subsequent lower blood concentrations may be attributed to the enhanced hepato-splenic and lung clearance of ligand anchored liposomes and entrapped Amp B.

Estimation of Amp B accumulated in various organ reveals that liposomal Amp B significantly alters the bio-distribution pattern of the free Amp B (Table 4). Although free Amp B itself accumulates in liver, spleen, lung and kidney, yet the rate, extent and duration of accumulation are significantly higher after the administration of liposomally entrapped Amp B. When compared amongst the respective groups, the difference was statistically significant ($P < 0.05$). The intravenous administration of the both the plain liposomal formulations exhibited higher rates and higher extent of drug uptake by liver, spleen and lungs with a concomitant reduction in drug accumulation in kidney.

Coating of liposomes with OPM further enhanced the accumulation of Amp B selectively in liver as compared against plain liposomes (from 56.1 ± 3.86 to 66.1 ± 4.7%), spleen (from 13.9 ± 1.4 to 17.1 ± 1.5%) and lungs from (4.2 ± 0.02 to 10.7 ± 0.2%). Similar results were obtained following the administration of PAM linked formulation, where uptake by liver, spleen and lungs was significantly higher than respective plain (non-ligand anchored) formulations. The enhanced accumulation of Amp B was recorded specially in liver as compared with plain liposomes (from 53.2 ± 2.3 to 70.8 ± 2.5%), spleen (from 9.8 ± 0.4 to 17.3 ± 1.1%) and lungs from (4.9 ± 0.03 to 6.9 ± 0.3%).

The hepato-splenic quantitative uptake of OPM coated and PAM linked liposomes were lowered when 10.0 mg hydrolysed mannan was administered intravenously prior to injecting the formulations (Fig. 5A and B). This qualitatively

Table 4
Organ distribution of Amp B following administration of various formulations[a]

Codes	Organ	Percent dose recovered after					
		15 min	30 min	1 h	2 h	4 h	24 h
Free Amp B							
	Liver	21.6 ± 1.3	28.7 ± 1.7	35.6 ± 1.1	30.1 ± 1.4	19.8 ± 0.7	4.7 ± 0.1
	Spleen	4.6 ± 0.01	5.8 ± 0.05	7.1 ± 0.01	5.9 ± 0.08	4.6 ± 0.04	1.2 ± 0.05
	Lung	2.3 ± 0.01	3.8 ± 0.01	2.7 ± 0.01	2.1 ± 0.01	0.9 ± 0.01	ND[b]
	Kidney	6.8 ± 0.01	4.3 ± 0.01	2.1 ± 0.01	1.1 ± 0.01	ND[b]	ND[b]
PC3							
	Liver	36.9 ± 0.1	51.4 ± 4.7	56.1 ± 2.7	49.2 ± 1.8	46.1 ± 1.1	39.1 ± 1.5
	Spleen	8.1 ± 0.01	9.8 ± 0.01	13.9 ± 0.1	12.3 ± 0.1	10.3 ± 0.1	8.2 ± 0.01
	Lung	3.1 ± 0.01	3.9 ± 0.01	4.2 ± 0.02	4.1 ± 0.07	2.9 ± 0.01	0.9 ± 0.01
	Kidney	ND[b]	ND[b]	1.2 ± 0.02	2.3 ± 0.01	1.8 ± 0.01	ND[b]
OPM coated PC_3							
	Liver	42.1 ± 1.6	58.6 ± 2.8	66.1 ± 3.1	63.4 ± 2.7	60.7 ± 4.1	53.8 ± 3.1
	Spleen	12.4 ± 0.7	15.9 ± 1.6	17.1 ± 1.1	15.1 ± 0.9	14.2 ± 0.5	10.1 ± 0.6
	Lung	7.9 ± 0.3	9.1 ± 0.1	10.7 ± 0.2	10.9 ± 0.7	8.1 ± 0.09	4.6 ± 0.02
	Kidney	ND[b]	ND[b]	1.1 ± 0.3	1.9 ± 0.01	1.6 ± 0.01	ND[b]
CE3							
	Liver	31.8 ± 1.1	46.1 ± 1.2	53.2 ± 2.3	48.1 ± 1.9	44.3 ± 3.1	31.9 ± 1.2
	Spleen	6.3 ± 0.8	7.9 ± 0.1	9.8 ± 0.4	8.6 ± 0.1	7.5 ± 0.4	3.9 ± 0.2
	Lung	2.1 ± 0.2	3.8 ± 0.2	4.9 ± 0.03	3.6 ± 0.01	3.1 ± 0.3	1.4 ± 0.09
	Kidney	ND[b]	0.8 ± 0.04	1.3 ± 0.01	2.8 ± 0.02	4.3 ± 0.1	0.9 ± 0.01
Mannopyranoside linked CE3							
	Liver	47.9 ± 2.5	65.1 ± 2.7	70.8 ± 2.5	61.4 ± 2.9	58.3 ± 2.8	51.1 ± 3.2
	Spleen	14.4 ± 1.5	16.1 ± 1.1	17.3 ± 1.1	19.6 ± 1.1	17.4 ± 1.1	11.2 ± 1.1
	Lung	5.1 ± 0.2	6.2 ± 0.4	6.9 ± 0.3	5.8 ± 0.4	3.6 ± 0.02	1.8 ± 0.1
	Kidney	ND[b]	ND[b]	0.6 ± .001	1.8 ± 0.03	2.7 ± 0.01	ND[b]

[a] All the values are representatives of mean ± S.D. for three independent determinations.
[b] ND, not detected.

suggests the possible involvement of mannose receptors expressed onto the membrane of macrophages. The later may be involved in the selective and higher uptake of the ligand appended liposomes. The faster rates and higher extent of liposome uptake by liver, spleen and lungs on anchoring mannose terminated ligands to them has been reported by various workers (Ghosh and Bacchawat, 1980; Szoka and Mayhew, 1983; Das et al., 1985). However, the increased macrophage uptake is not completely inhibited by the pre-injection of hydrolysed mannan; which suggests that some other mechanisms besides mannose/fucose receptor mediated endocytosis are also involved in promoting and contributing to an enhanced macrophage uptake. We speculate that probably uptake via anionic scavenger receptor mediated endocytosis is in part responsible for the observed higher uptake. This hypothesis, however, need confirmation through the competitive inhibition studies.

Comparison of the bio-distribution patterns after the administration of OPM coated and PAM anchored liposomes suggest that both the formulations exhibited higher accumulation levels in liver and spleen as compared with plain liposomes containing an equivalent dose of Amp B. Significant ($P < 0.05$ in a rank sum test) statistical difference in the resulting bio-distribution patterns exclude the probability of inter-/intra-subject variations substantiating the role of ligand–receptor

interaction mediated phenomenon. However, the relative hepato-splenic accumulation of the Amp B entrapped in the PAM anchored liposomes was higher. The results of bio-distribution studies also suggest that OPM coated liposomes are more effective than mannopyranoside linked liposomes in regard to targeting bioactives to the lungs as the accumulation of OPM coated formulation was quantitatively higher (10.7 ± 0.2) than that of PAM linked formulation (6.9 ± 0.3) in lungs. The analysis of the data obtained from biodistribution studies (in the form of drug localisation indices, Fig. 6) reveals that in the case of liver and spleen the indices remained significantly higher over 24 h for both ligand appended formulations. These findings help conclude that considerably higher concentrations of the drug could be maintained in the organs over the protracted period of time. On comparing the drug localisation indices of ligand appended formulations it is seen that drug localisation indices in liver and spleen are higher in the case of PAM linked formulation than in the case of OPM coated formulation. In the case of lungs however, OPM coated liposome was found to exhibit a higher and better drug localisation index. The observed values suggest that the ligand anchored liposomes are not only effective in rapid attainment of high drug concentrations in macrophage rich organs but also maintain the concentration levels over a prolonged period of time, when compared against the free drug. This establishes the significance of the targeting potential of the developed systems.

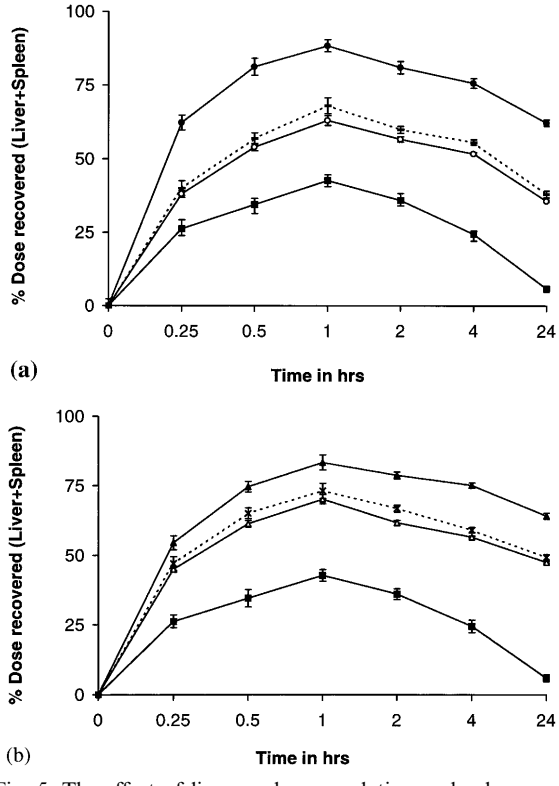

Fig. 5. The effect of liposomal encapsulation and subsequent ligand anchoring on the hepato-splenic uptake of Amp B. Total percent dose recovered from liver and spleen has been recorded as a function of time and compared with the free drug (◆). Liposomal encapsulation (▲) enhanced the accumulation which was further enhanced on anchoring ligands (●), mannopyranoside (A) or coating OPM (B) on to the liposomal surface. Enhanced uptake was competitively inhibited on pre-injection of hydrolysed mannan (○).

4. Conclusion

The developed systems (mannose terminating ligand-anchored liposomes) appear promising for the treatment of hepato-splenic candidiasis and leishmaniasis specifically. Furthermore, in systemic fungal infections the systems can be used for rapid loading of RES organs with the incorporated drug for the complete eradication of the intracellular pathogens. It is also concluded that targeting profile of OPM coated liposomes could be utilised for targeting Amp B to lung tissues in pulmonary fungal infections and similarly other bioactives in the treatment of infectious diseases of respiratory tract. Bio-response modifiers can also be incorporated in such systems for achieving effective macrophage activation that can serve as powerful synergistic effect to the therapy of opportunistic infections.

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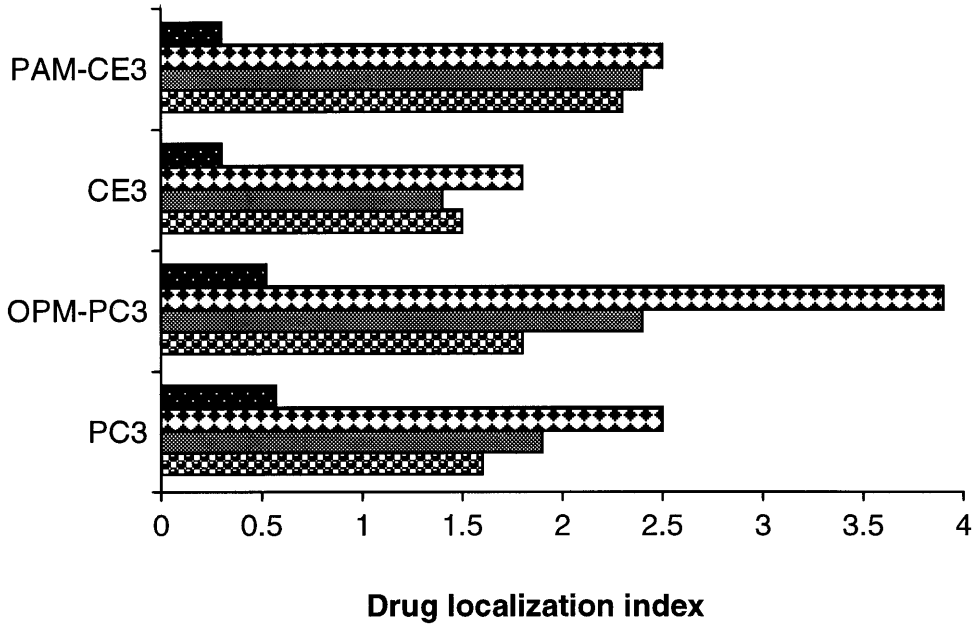

Fig. 6. Drug localisation indices for different organs recorded after 1 hour with different formulations. Drug localisation index for liver (▩), spleen (▨), lung (▩) and kidney (■) were calculated using the data from organ distribution studies by the formula given by Gupta and Huang (1989).

References

Adamson, A.W., 1967. Physical Chemistry of Surface, II ed. Interscience, New York, p. 223.

Alving, C.R., 1982. Therapeutic potential of liposomes as drug carriers in leishmaniasis, malaria and vaccines. In: Gregoriadis, G., Senior, J., Trouet, A. (Eds.), Targeting of Drugs. Plenum Press, New York, pp. 285–312.

Bachhawat, B.K., Das, P.K., Ghosh, P.K., 1984. Preparation of glycosides bearing liposomes fir targeting. In: Gregoriadis, G. (Ed.), Liposome Technology. CRC Press, Boca Raton, FL, pp. 117–138.

Bakker-Wouderberg, I.A., Storm, G., Woodle, M.C., 1994. Liposomes in the treatment of infections. J. Drug Targeting 2, 363–371.

Barrat, G.M., Tenu, J.P., Yapo, A., Petit, J.F., 1986. Preparation and characterization of liposomes containing mannosylated phospholipids capable of targeting drugs to macrophages. Biochim. Biophys. Acta 862, 153–156.

Brajtburg, J., Powderly, W.G., Kobayashi, G., Medoff, G., 1990. Amphotericin B: delivery systems. Antimicrob. Agents Chemother. 34, 381–384.

Das, P.K., Murray, G.J., Zairzow, G.C., Brady, R.O., Barranger, J.A., 1985. Lectin specific targeting of β-glucocerebrosidase to different liver cells via glycosylated liposomes. Biochem. Med. 33, 124–131.

de Marie, S., Janknegt, R., Bakker-Woundenberg, I.A.J.M., 1994. Clinical use of liposomal and lipid-complexed amphotericin B. J. Antimicrob. Chemother. 33, 907–916.

Denning, D.W., Stevens, D.A., 1990. Antifungal and surgical treatment of invasive fungal aspergillosis: review of 2121 published cases. Rev. Infect. Dis. 12, 1147–1201.

Fidler, I.J., 1988. Targeting of immunomodulator to mononuclear phagocytes for the therapy of cancer. Adv. Drug Deliv. Rev. 2, 69–106.

Garcon, N., Gregoriadis, G., Taylor, M., Summerfield, J., 1988. Mannose mediated targeted immunoadjuvant action of liposomes. Immunology 64, 743–748.

Ghosh, P., Bacchawat, B.K., 1980. Grafting of different glycosides on the surface of liposomes and its effects on the tissue distribution of 125I labelled γ-globulin encapsulated in liposomes. Biochim. Biophys. Acta 632, 560–565.

Ghosh, P., Das, P.K., Bacchhawat, B.K., 1982. Targeting of liposomes towards different cell types of rat liver through the involvement of liposomal surface glycosides. Arch. Biochim. Biophys. 213, 266–270.

Gordon, S., Rabinowitz, S., 1989. Macrophages as targets for drug delivery. Adv. Drug Deliv. Rev. 4, 27–47.

Gupta, P.K., Huang, C.T., 1989. Albumin microspheres I — physicochemical characterstics. J. Microencapsulation 6, 427–462.

Hammerling, U., Westphal, O., 1967. Synthesis and use of *O*-stearoyl polysaccharides in passive hemagglutination and hemolysis. Eur. J. Biochem. 1, 46–50.

Hiemenz, J.W., Walsh, T.J., 1996. Lipid formulations of amphotericin B: recent progress and future directions. Clin. Infect. Dis. 22 (S-2), S133–S144.

Iwamoto, K., Kato, T., Mosahiro, K., Koyama, N., Watanabe, S., Miyake, Y., Sunamoto, G., 1991. Polysaccharide coated oil droplets in O/W emulsions as targetable carriers for lipophilic drugs. J. Pharm. Sci. 80, 214–219.

Jaitely, V., Vyas, S.P., 1999. Development and characterization of supramolecular autovectoring system for selective drug delivery. J. Drug Targeting 6, 315–322.

Janknegt, R., de Marie, S., Bakker-Woundenberg, I.A.J.M., Crommellin, D.J., 1992. Liposomal and lipid formulations of amphotericin B. Clinical pharmacokinetics. Clin. Pharmacokinet. 23, 279–291.

Litzinger, D.C., Huang, L., 1992. Phosphatidylethanolamine liposomes: drug delivery, gene transfer and immunodiagnostic applications. Biochim. Biophys. Acta 1113, 201–227.

Lopez-Berestein, G., Mehta, R., Hopffer, R., Mills, K., Kasi, L., Mehta, K., Fainstein, V., Luna, M., Harsh, E.N., Juliano, R., 1983. Treatment and prophylaxis of disseminated infections due to *Candida albicans* in mice with liposomal encapsulated amphotericin B. J. Infect. Dis. 147, 939–945.

Mehta, R., Lopez-Berehtein, G., Hopffer, R., Mills, K., Juliano, R.L., 1984. Liposomal amphotericin B is toxic to fungal cells but not to mammalian cells. Biochim. Biophys. Acta 770, 230–234.

New, R.R.C., 1990. Characterization of liposomes. In: Liposomes: A Practical Approach. Oxford University Press, Oxford, pp. 125–126.

O'Mullane, J.E., Artursson, P., Tomlinsson, E., 1987. Biopharmaceutics of microparticulate drug carriers. In: Juliano, R.L. (Ed.), Biological Approaches to the Controlled Delivery of Drugs. Ann. New York Acad. Sci. 507, 120–140.

Pavia, D.L., Lampman, G.L., Kriz, G.S., 1979. Infrared absorption process. In: Hoit, G., Rinehart, K.L., Weinstein, I.B. (Eds.), Introduction to Spectroscopy: A guide for Students of Organic Chemistry. Saunder's College, Philadelphia, pp. 13–15.

Ponpipon, M.M., Shen, T.Y., Baldeshwieler, J.D., Wu, P.S., 1984. Modification of liposomal surface properties by synthetic glycolipids. In: Gregoriadis, G. (Ed.), Liposome Technology, vol. 3. CRC Press, Boca Raton, FL, pp. 95–125.

Sarosi, G.A., 1990. Amphotericin B: still the 'gold standard' for antifungal therapy. Postgrad. Med. 88, 151–161.

Sunamoto, J., Gato, M., Iida, T., Hara, K., Saito, A., Tomonega, A., 1985. Polymer coated liposomes for drug delivery to target specific organs. In: Gregoriadis, G., Senior, J., Trouet, A. (Eds.), Receptor Mediated Targeting of Drugs. Plenum Press, NewYork, pp. 359–371.

Szoka, F.C., Jr, Mayhew, E., 1983. Alteration of liposome disposition in vivo by bilayer situated carbohydrates. Biochim. Biophys. Res. Commun. 110, 140–147.

Van Etten, E.W.M., van den Heuvel-de Groot, C., Bakker-Wouderberg, I.A.J.M., 1993. Efficacies of amphotericin B-desoxycholate (Fungizone), liposomal amphotericin B (AmbiSome) and flucanazole in the treatment of systemic candidosis in immunocompetent and leucopenic mice. J. Antimicrob. Chemother. 32, 723–729.

Van Etten, E.W.M., OtteLambillion, M., van Vianen, W., tenKate, M.T., Bakker-Wouderberg, I.A.J.M., 1995. Biodistribution of liposomal amphotericin B (ambisome) and amphotericin B desoxycholate (Fungizone) in uninfected immunocompetent mice and leucopenic mice infected with *Candida albicans*. J. Antimicrob. Chemother. 35, 509–515.

van Rooijen, N.V., Sanders, A., 1994. Liposome mediated depletion of macrophages: mechanism of action, preparation of liposomes and applications. J. Immunol. Method 174, 83–93.

Vyas, S.P., 2000. Macrophage targeting and colloidal drug carriers. In: Vyas, S.P., Dixit, V.K. (Eds.), Advances in Liposomal Research. CBS Publishers and Distributors, New Delhi, pp. 98–120.

Walsh, T.J., Pizzo, P.A., 1990. Treatment of systemic fungal infections: recent progress and current problems. Eur. J. Clin. Microbiol. Infect. Dis. 7, 460–475.

Yachi, K., Kikuchi, K., Yamauchi, H., Hirota, S., Tomikawa, M., 1995. Distribution of liposomes containing mannobiose esters of fatty acids in rats. J. Microencapsulation 12, 377–388.