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# Mannosylated liposomes for bio-film targeting

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**Abstract**

Vesicular systems in general are investigated to achieve bacterial bio-film targeting as their architecture mimics bio-membranes in terms of structure and bio-behavior. This paper elaborates upon the role of the inherent characteristics of the carrier system and further envisages the role of anchored ligands in navigating the contents in the vicinity of bio-films. Vesicles in the present study were coated with hydrophobic derivatives of mannan (cholesteryl mannan and sialo-mannan). The prepared vesicles were characterized for size, shape, percentage entrapment and ligand binding specificity and results were compared with the uncoated versions. Using a set of in vitro and in vivo models, the bio-film targeting potential of plain and mannosylated liposomal formulations were compared. Results suggested that mannosylated vesicles could be effectively targeted to the model bacterial bio-films, compared with plain vesicles. Moreover, the sialo-mannan coated liposomes recorded superior targetability as reflected in the significantly higher percentage growth inhibition when compared with cholesteryl mannan coated liposomes. The engineered systems thus have the potential use for the delivery of anti-microbial agents to the bio-films.


*Keywords:* Bio-film; Liposomes; Mannosylated liposomes; Cholesteryl mannan; Sialo-mannan

## 1. Introduction

Research issues have been redefined and revolutionized by the fact that most bacteria in the bio-environment aggregate as bio-films. This is a growth domain in which bacteria behave very differently from free-floating (fluid phase, planktonic) bacteria growing in laboratory cultures. Bio-films can be considered as microbial ecosystems representing different microbial strains and species in aggregation, which efficiently co-ordinate and co-operate to protect themselves against environmental stresses and facilitate nutrient uptake for survival. They are layer-like aggregates and stable synergistic consortia of microorganisms attached to the surface of bio-materials and biological sites and demonstrate resistance towards conventionally applied anti-microbial agents. The resistance of these bio-films to anti-microbials is a constraint for the development of an acceptable dosage regimen. Not only do bio-films resist anti-microbials, but they also are large enough to defeat the immune system (Vyas and Sihorkar, 1999; Vyas et al., 2000a; Sihorkar and Vyas, 2001).

Drug delivery and targeting to the bacterial bio-films has received much interest. The potential of drug delivery through the localization and/or targeting of bio-films still remains to be proved and adopted in the field of pharmaceutical research. Liposomes are versatile drug carrier systems that have great potential in the treatment of infectious diseases due to protection of encapsulated drug in the biological milieu and transportation of the same to target specific sites. Liposomes have been investigated to achieve bacterial bio-film targeting as they mimic the bio-membranes in terms of structure and bio-behavior. Targeting could be achieved due to intrinsic and inherent distribution of carrier (passive targeting) or by engineering surface navigator molecules (ligands) to alter its distribution or uptake in the biological milieu and to release the drug in the proximity of bacterial bio-films. Liposome targeting to some strains of skin-associated bacteria, bacteria of periodontal pockets and the oral cavity has been investigated using a number of different approaches. These include lectinized liposomes, polysaccharide coated liposomes,

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Table 1
Compositions and different in vitro characteristics of the developed formulations

| Composition | Code | Lipid molar ratio | Lipid:ligand (w/w) | Average mean diameter | Poly-dispersity index | % Entrapment |
|---|---|---|---|---|---|---|
| Before extrusion | | | | | | |
| PC:Chol | PL-1 | 7:3 | – | 2.67 ± 0.6 μm | 0.812 | 29.6 ± 3.8 |
| PC:Chol:SA | PL-2 | 7:2:1 | – | 3.12 ± 0.4 μm | 0.664 | 38.6 ± 2.2 |
| After extrusion | | | | | | |
| PC:Chol:SA | EL | 7:2:1 | – | 412 ± 61 nm | 0.092 | 33.1 ± 2.8 |
| (PC:Chol:SA): CHM | EL-CHM | 7:2:1 | 5:1 | 454 ± 42 nm | 0.124 | 31.7 ± 2.5 |
| (PC:Chol:SA): SM | EL-SM | 7:2:1 | 7:1 | 467 ± 51 nm | 0.143 | 30.9 ± 3.1 |

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

cationic liposomes and monoclonal antibodies coated liposomes (Nacucchio et al., 1988; Hutchinson et al., 1989; Jones et al., 1994a,b, 1997; Robinson et al., 1998; Vyas et al., 2001; Jones, 2005).

The work envisaged makes use of the inherent characteristics of the carrier (liposomes) and anchored ligands (mannan) to mediate receptor-mediated uptake of the contents into the vicinity of the bio-films. In the present study, we prepared an antibacterial drug (metronidazole)-loaded mannosylated liposomes anchored with different mannan derivatives, namely, cholesteryl mannan (CHM) and sialo-mannan (SM). The targeting efficiency in terms of percentage bacterial growth inhibition of both in vitro and in vivo bacterial bio-films was investigated.

## 2. Materials and methods

### 2.1. Materials

Egg phosphatidylcholine (EPC), stearylamine (SA), cholesterol (Chol), mannan, concanavalin A (Con-A), wheat germ agglutinin (WGA), Triton X-100 and Sephadex G-50 were purchased from Sigma Chemicals Co. (USA). 1-Ethyl-3-(3-dimethylamino) propylcarbodimide, cholesteryl chloroformate and ethylenediamine were procured from Fluka, Switzerland. The drug Metronidazole was a gift sample from MAC Laboratories Ltd., Mumbai, India. Other materials and reagents were of analytical grade (Qualigens, Chemical division of Glaxo India Ltd.).

### 2.2. Preparation of plain liposomes

Liposomes were prepared by the classical cast film technique. Lipid constituents were taken in different mole fraction ratio (Table 1) and dissolved in minimum quantity of chloroform:methanol mixture (7:3, v/v) in a round bottom flask. The organic solvent was evaporated under reduced pressure using Buchi rotary flash evaporator to cast the thin lipid film on the walls of the round bottom flask. The lipid film was hydrated with 5 ml of phosphate buffer saline (PBS 0.01 M, pH 7.4) containing 1 mg/ml of metronidazole at room temperature. The dispersion was passed through Sephadex G-50 column to remove unentrapped drug. These vesicles were subjected to pre-filtration (twice) through a large pore size filter (1.0–2.0 μm). Properly diluted pre-sized vesicles were then extruded 10 times through two-stacked Nucleopore polycarbonate filter of 0.4 μm-pore diameters by applying a pressure gradient (∼120 psi) with a mini-extruder fitted with two 5 ml syringes. The dispersion was kept above $T_c^\circ$ of constituent lipids at the time of extrusion. The harvested and screened liposomal populations were taken for ligand-anchoring and subsequent studies.

### 2.3. Synthesis of mannan derivatives

Cholesterol derivative of mannan (Cholesteryl mannan, CHM) was synthesized following the procedure earlier reported by our group (Sihorkar and Vyas, 1999). Cholesteryl mannan (CHM) was used to further synthesize sialo-mannan (SM). *N*-Acetyl neuraminic acid was introduced to CHM according to the method described by Kijima et al. (1982) and Mollerfield et al. (1986) with minor modifications. Briefly, neuraminic acid (1.0 g, 3.2 mmol) was reacted with acetyl chloride (50 ml, 0.64 mol) under stirring at room temperature for 48 h. Gaseous dry HCl was bubbled into the reaction mixture for 24 h at room temperature. Unreacted acetyl chloride and HCl were removed under reduced pressure to obtain 4,7,8,9-tetra-2-chloro-*N*-acetyl neuraminic acid. This neuraminic acid derivative (397 mg, 0.8 mmol) was reacted with CHM (130 mg, 0.8 mmol) in a mixture of 10 ml dry dimethyl sulfoxide (DMSO) and 5 ml of dry dimethyl formamide (DMF). The reaction mixture was stirred under nitrogen atmosphere for 4 days at 4 °C in dark. The precipitate obtained was separated and the filtrate was poured into 100 ml ethanol. The resultant solution was kept overnight. This process yielded a precipitate, which was separated and redissolved in 50 ml distilled water. The resultant solution was then deacetylated with 20 ml of 1 mol $dm^{-3}$ NaOH for 30 min in an ice bath, neutralized with 1 mol $dm^{-3}$ HCl and then finally dialyzed against distilled water for 48 h to yield sialic acid substituted mannan (SM). The derivatized product was subjected to characterization using IR and $^1$H NMR spectroscopy.

### 2.4. Coating of liposomes with mannan derivatives

Anchoring of liposomes with hydrophobized mannan derivatives (CHM and SM) was accomplished following the procedure developed in our laboratory (Vyas et al., 2000b; Jain and Vyas, 2005; Jain et al., 2005). In brief, a 2 ml of uncoated liposomal formulation was incubated with CHM/SM dispersion (in PBS, pH 7.4) and the mixture was stirred gently at room temperature. After completion of coating the excessive unbound ligands were removed by spinning the resultant suspension

through a Sephadex G-50 column at 2000 rpm for 5 min. The two process variables (total lipid to ligand weight ratio and incubation time) were optimized by measuring the change in zeta potential of the dispersion (Zetasizer 3000 HS, Malvern Instruments Co., UK). During the coating process, hydrophobic mannan derivatives get coated on to the positively charged liposomes, thereby reducing the inherent charge on the vesicles. This change in zeta potential value was used to optimize the process variables. For optimization of total lipid to ligand ratio, formulations with different ratios were prepared and incubated for a fixed time period of 24 h. The optimum ratio was determined at which no significant change in zeta potential was recorded on further increasing the lipid to ligand ratio. Similarly, for optimization of incubation time the formulations with optimum lipid to ligand ratio were prepared and incubated for different time periods and their zeta potential measured. After completion of the coating no significant change in zeta potential was recorded.

### 2.5. In vitro characterization

Developed formulations were characterized in vitro prior to and after surface ligand anchoring. Formulations were evaluated for their shape and morphology by phase contrast microscopy (Leitz-biomed, Germany) and transmission electron microscopy (TEM) after negative staining using phosphotungstic acid (1%) (Philips, Japan). The mean vesicle size was determined using laser diffraction based particle size analyzer (CILAS 1064, France). The percent entrapment was determined and expressed as the percentage fraction of the drug incorporated in the vesicles. Vesicles were lysed with a minimum amount of Triton X-100 (0.5%, v/v), centrifuged and the liberated drug (metronidazole) was analyzed at 274 nm using a Shimadzu 1601 DB UV/VIS spectrometer (Japan) against reagent blank.

### 2.6. Ligand binding specificity of liposome anchored ligands

The developed systems (CHM and SM-anchored) were assessed for in vitro ligand-specific activity. The affinity towards exogenously provided lectins Concanavalin A (Con-A) and wheat germ agglutinin (WGA) was used as a measure of activity for CHM and SM anchored liposomes, respectively. A 200 μl sample of the original dispersions (plain and CHM anchored liposomes) was diluted 10 times with PBS (pH 7.4) and 1 ml of Con A (1 mg/ml) in PBS (pH 7.4) with 5 mM of calcium chloride and 5 mM of magnesium chloride was added to it. The increase in turbidity at 550 nm was monitored spectrophotometrically (Shimadzu 1601 DB UV/VIS spectrophotometer, Japan) for 2 h. Similar experiments were performed taking WGA as the counter ligand for SM-anchored liposomes and turbidity increase was recorded at 550 nm.

### 2.7. Models for bio-film targeting assay

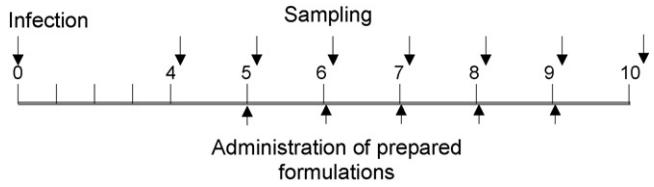
Ligand-anchored vesicular constructs were further investigated for their bio-film targeting potential against *Staphylococcus aureus* bio-films both in vitro and in vivo. A microtitre plate bio-film model of *S. aureus* was developed and used in our study as an in vitro model to assess the efficacy of the formulations using a regrowth assay (Sihorkar and Vyas, 2001). An in vivo bio-film model of *S. aureus* was also developed using a rat pouch infected site and formulations were assessed in terms of their ability to offer reduction in the percentage bacterial viability (Kawashima et al., 1993).

#### 2.7.1. In vitro microtitre plate model

In this model *S. aureus* bacterial strips (MTCC 740, IMT, Chandigarh, India) were used to inoculate agar plates prepared from brain heart infusion (BHI, 3.7 g) in double distilled water (100 ml) to which bacteriological agar (1.5 g) was added. The mixture was boiled to dissolve the agar and sterilized by autoclaving. The sterile plates were inoculated by streaking and the inverted streaked plates were incubated at 37 °C for 18 h. The resulting colonies were used to inoculate aliquots (10 ml) of previously sterilized nutrient broth prepared by mixing BHI (3.7 g) and yeast extract powder (0.39 g) in double distilled water (100 ml). These were incubated in capped bottles at 37 °C for 18 h after which the bacterial suspensions were centrifuged (200 rpm, 15 min), the supernatant was discarded and the separated pellets were resuspended in sterile PBS. The centrifugation and re-suspension were repeated thrice and the bacterial cell concentration appropriately adjusted by dilution with phosphate buffer to give an absorbance of 0.5 at 550 nm. A 100 μl of this final bacterial suspension was transferred into microtitre plates and left overnight at room temperature for the cells to adhere. Before use the plates were washed three times with sterile phosphate saline buffer (pH 7.4). To measure the targetability in terms of percentage bacterial growth inhibition (% BGI), microtitre plates of bacterial bio-films were incubated with different liposomal formulations (200 μl) at various drug concentrations (50%, 75% and 90% of MIC of metronidazole) at 37 ± 1 °C for definite time interval (60 min). After incubation, the plates were washed thrice with sterile PBS (pH 7.4) and blotted dry. A regrowth assay was performed to check the specificity and targetability of the developed system against *S. aureus* bio-films. Sterile nutrient broth (200 μl) was added to each well and the plates were incubated for 24 h at 37 ± 1 °C in a candle jar. After 24 h the absorbance of each well was measured at 550 nm using a plate reader. The increase in absorbance was taken as a measure of bacterial growth. The absorbance of wells containing only bacteria and no bacteria (after 24 h exposure to growth media) was taken as 100% and 0% growth, respectively. In a parallel experiment, the effect of metronidazole on suspension culture (fluid phase) of *S. aureus* was also tested maintaining the same experimental condition except the phase (bio-film phase versus fluid phase).

#### 2.7.2. In vivo CMC pouch infection model

The model has been reported to investigate the efficacy of antibiotics and their interaction with in vivo produced bio-films (Kawashima et al., 1993; Nomura et al., 2002). The study was carried out under the guidelines compiled by CPCSEA (Committee for the Purpose of Control and Supervision of Experi-




Scheme 1. Experimental schedule for the viability assessment of bacteria in bio-film of *S. aureus*.

ments on Animal, Ministry of Culture, Government of India) and all the study protocols were approved by local Institutional Animal Ethics Committee. An air pouch on the back of albino rats (either sex, weighing 100–150 g) was formed by injecting 1.0 ml of air subcutaneously with a 21-gauge needle after trimming off the hair with a hair clipper. Just after removal of the needle, the needle hole in the skin was sealed with an adhesive agent. The next day, under anesthesia, the air in the pouch was aspirated and 1.0 ml of sterilized CMC (1.5% in saline) was injected to form a carboxymethylcellulose (CMC) pouch. An infection was induced by inoculating $10^6$ colony forming units (CFU) of *S. aureus* per pouch along with the injection of CMC.

The therapy with drug (control), plain and ligand-anchored liposomes began 4 days after the infection with local administration of different formulations in a dose equivalent to 90% MIC of metronidazole. The administration of different systems was continued once a day for 5 days, except for the plain drug, which was administered twice a day as per its normal dosage regimen. Pouch exudates were sampled with the help of a syringe once a day for 7 days starting from the day 4. The numbers of viable bacteria in the pouch exudates were counted using established procedures. The experimental protocol designed is depicted in Scheme 1.

### 2.8. Statistical analysis

The results were expressed as mean ± standard deviation (S.D.). One way analysis of variance (ANOVA) followed by post hoc test (Tukey's test) was used to evaluate the effect of lipid to ligand ratio or incubation time and zeta potential ($n = 6$). The effect of incubation time with lectin (Con-A or WGA) and liposomes type on optical density was analyzed using repeated measures ANOVA followed by Tukey's test ($n = 6$). Analysis of % BGI values (in vitro microtitre plate model) and % viable bacterial count (in vivo CMC pouch model) recorded following treatment with different doses of developed formulations was also performed using two-way ANOVA followed by Tukey's test ($n = 6$). Statistical significance was designated as $P < 0.05$.

## 3. Results and discussion

### 3.1. Preparation and optimization of mannosylated liposomes

The aim of the study was to exploit the inherent characteristics of the carrier and anchored ligands to deliver the contents into the vicinity of the bio-films. For this purpose, various mannosylated liposomes were studied for their selectivity in targeting and efficiency in delivery of loaded drug to the bio-films. Since mannan is an extremely hydrophilic polysaccharide, it cannot be coated onto the lipoidal vesicular surface by simple incubation. Therefore, it was necessary to chemically modify the mannan by conjugating it to a hydrophobic anchor, which allows the polysaccharide to interdigitate with the vesicle membrane. The synthesis of CHM and SM was confirmed by IR and NMR analysis. The derivatization procedure employed to synthesize CHM gave a degree of substitution of 0.71 cholesterol molecules per hundred saccharide units of mannan as estimated by $^1$H NMR. Hydrophobic anchors of the synthesized mannan derivatives interact with the outer half of the liposomal bilayer during the coating process thus orienting and projecting hydrophilic portion towards the aqueous bulk. In this way a two dimensional network of polymers is formed around the liposomal membranes. Initial experiments were conducted to optimize the procedure of ligand anchoring. The parameters studied include lipid to ligand ratio and incubation time.

For optimization of total lipid to ligand ratio in order to achieve maximum coating, formulations using different ratios (ranging from 10:1 to 1:1, w/w) were prepared and zeta potential was determined (Fig. 1). The initial positive value of zeta potential decreased on increasing the ratio of anionic polysaccharides (CHM and SM) and approached towards a minimum value at 5:1 and 7:1 lipid to ligand weight ratio respectively. It apparently relates to the extent of charge masking effect of anionic polysaccharides. On further addition of ligands especially beyond these optimum ratios no significant change in zeta potential was recorded ($P > 0.05$). It indicates that at 5:1 lipid to CHM ratio and 7:1 lipid to SM ratio, the integration and interdigitation of respective ligand with liposomal membrane could have been completed (Jain and Vyas, 2005; Jain et al., 2005).

Similarly, for optimization of incubation time the formulations using optimum lipid to ligand ratio were prepared and incubated for different incubation time periods, viz. 0, 1, 2, 3, 4, 6, 8, 12 and up to 24 h and the change in zeta potential was recorded (Fig. 2). The zeta potential values fell down steeply from their initial values, which might be attributed to the charge

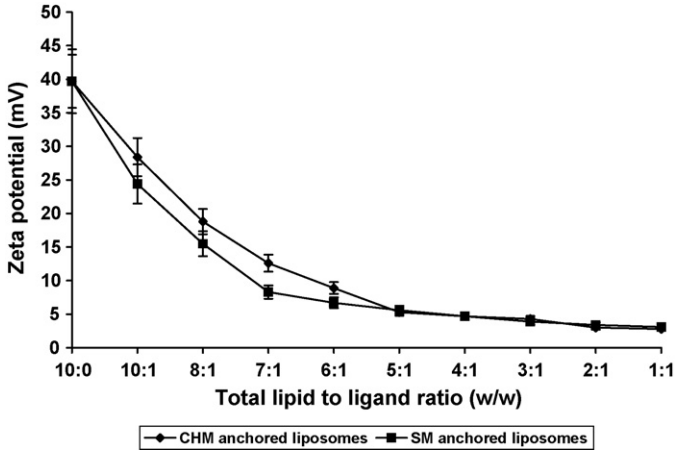



Fig. 1. Optimization of lipid to ligand weight ratio. Values are expressed as mean ± S.D. ($n = 6$).

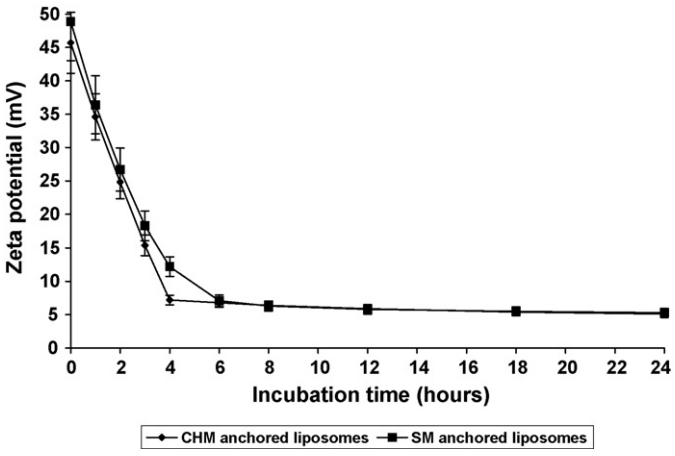



Fig. 2. Optimization of incubation time for complete coating. Values are expressed as mean ± S.D. ($n = 6$).

quenching of surface associated free ligand. With longer incubation times (4 h in case of CHM anchored liposomes and 6 h for SM anchored liposomes), the amount of residual free ligand decreased and the change in zeta potential was not significant ($P > 0.05$). This shows that at the end of 4 and 6 h, the integration and interdigitation of added ligands with lipid bilayers might have been completed (Jain and Vyas, 2005; Jain et al., 2005).

### 3.2. In vitro characterization

Plain liposomes and those anchored with hydrophobized mannan (CHM and SM) were subjected to vesicle shape, size analysis and percent entrapment measurements at appropriate steps. Results are summarized in Table 1. The shape of the vesicles prior to extrusion was observed under a phase contrast optical microscope (Leitz-Biomed, Germany) and was found to be spherical and multi-lamellar irrespective of the lipid composition. These multi-lamellar vesicles were extruded through polycarbonate filters of 0.4 μm-pore diameter. The extruded vesicles were found to be uni-lamellar as observed under TEM. The significant higher entrapment ($P < 0.05$) recorded with the incorporation of stearylamine (38.6 ± 2.2%) as compared against uncharged formulation (29.6 ± 3.8%) could be ascribed to an increase in the inter-lamellae distance and entrapped aqueous volume of vesicles. These inter-lamellae spaces may induce repulsion amongst the bilayers and hence leads to higher entrapment values (New, 1990). Extrusion of vesicles through 0.4 μm polycarbonate membrane filter resulted in significant reduction in entrapment efficiency ($P < 0.05$) due to possible leakage of drug during extrusion process under pressure. Extruded vesicles showed mean entrapment efficiency of 33.1 ± 2.8%. Percent drug entrapment in CHM and SM anchored liposomes was 31.7 ± 2.5 and 30.9 ± 3.1%, respectively, revealing that CHM and SM anchoring didn't result in significant reduction in the entrapment efficiency compared to plain uncoated extruded liposomes ($P > 0.05$). Preformed hydrated vesicles were used for anchoring of ligands and this may presumably be the reason for the insignificant changes recorded in the percentage entrapment values after coating.

Mean vesicle size of plain liposomes before extrusion was found to be 2.67 ± 0.6 μm (PC:Chol based formulation) and 3.12 ± 0.4 μm (PC:Chol:SA based formulation). The charge-induced repulsion between the bilayers may also accounted for larger size of stearylamine based formulations (New, 1990). Vesicles within a size range of 400–450 nm were obtained after extrusion. Polydispersity values demonstrated that following extrusion, acceptable dispersion size homogeneity (index ranging from 0.09 to 0.14) could be achieved, while the vesicles before extrusion recorded polydispersity values approaching towards the "cut off" range (∼0.7) of the apparatus indicating increase in heterogeneity.

Lectin-induced aggregation of the mannan-coated liposomes was assessed for the ligand activity and specificity assay. Concanavalin A (Con-A) is one of the well-investigated lectin and is known to specifically bind mannose, fructose and glucose residues. On the other hand, wheat germ agglutinin (WGA) specifically binds sialic acid terminus of the complex glycoproteins or carbohydrate structures (Rademacher et al., 1988; Palomino, 1994). A dramatic increase in the turbidity as monitored by optical density at 550 nm was observed in the case of dispersion containing mannosylated liposomes following exposure to lectins. In contrast, plain liposomes did not show any significant change ($P > 0.05$) in turbidity following exposure to lectin (Fig. 3a and b). The results suggested that mannan retained the binding specificity towards the lectins even after chemical modification and anchoring onto the liposome surface and they were oriented towards the aqueous bulk and were available for such interaction.

### 3.3. Bio-film targeting assay

Bacterial bio-films on the living tissues that result in chronic or refractory infections are probably constructed on mucosal or other bio-surfaces or bio-materials by way of complicating mechanisms. Many investigators reported in vitro experimental models of bacterial bio-films on the microtitre plates, silicone catheter tubing, cellulose acetate membrane and the surface of other devices by culturing bacteria in an artificial medium (Gottenbos et al., 1999; Sihorkar and Vyas, 2001). Ideal properties sought in these in vitro bacterial bio-film models are uniform film formation, uniform reproducibility and the ability to be investigated quantitatively.

*S. aureus* bio-films were prepared and taken as the model bio-film in our study. The Staphylococci develop as a plaque (bio-film) rather than as a fluid phase and behave rather differently in their pharmacological and metabolic manifestations. They secrete an extra-cellular polysaccharide 'slime' substance in which, the cells become embedded in to form a outer sheath that offer protection against conventional means to achieve therapeutic levels of the administered anti-microbial agent thus making their eradication difficult (Potera, 1996).

#### 3.3.1. In vitro microtitre plate model

For the targeting assay of in vitro bio-films of *S. aureus* with the developed systems, a modified microtitre plate model was selected. Bio-film was formed overnight on the microtitre plates

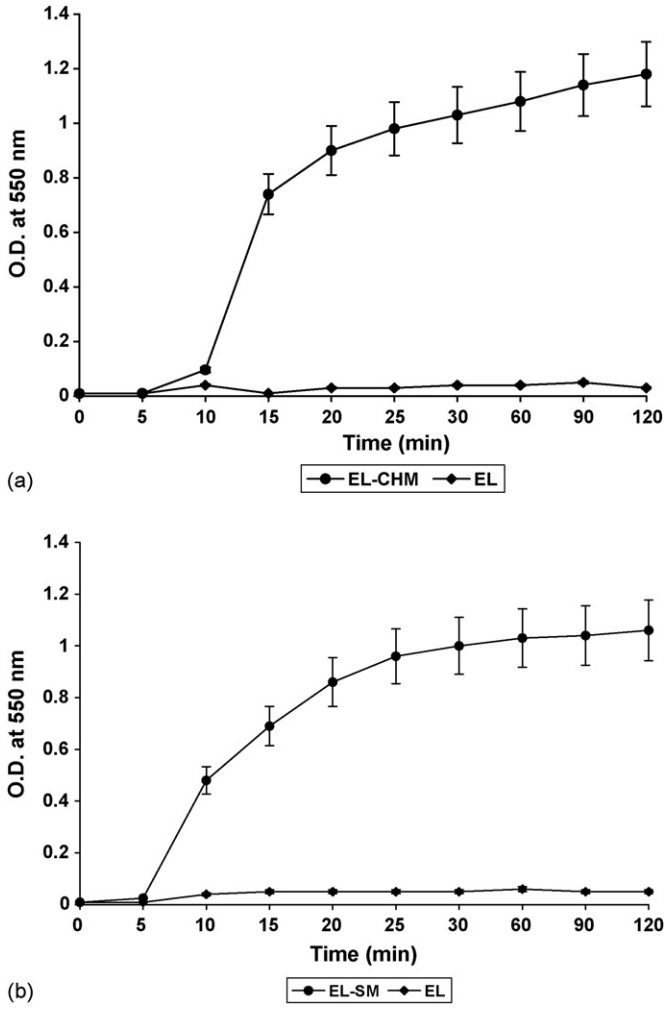



Fig. 3. In vitro ligand specificity of mannosylated liposomes by lectin induced aggregation method. (a) CHM anchored liposomes by Con-A induced aggregation; (b) SM anchored liposomes by WGA induced aggregation. Values are expressed as mean ± S.D. ($n = 6$).

and incubated with metronidazole-loaded vesicular dispersions along with controls. After disruption and washing of the bio-film-vesicle composites, a regrowth assay was performed. The efficiency of targeting of mannosylated liposomes to *S. aureus* bio-films has been measured in terms of % BGI, which served as an index of bio-film targeting.

Fig. 4 compares differences in the antibacterial activity of the same drug in same concentration but against bacteria harboring within two different phases. It could be seen that while in the fluid phase, metronidazole exhibited growth inhibition in a dose-dependent manner, whereas at the same dose-level same effect could not be achieved when tested against bacteria in the bio-film-phase. This may account for the drug resistance problems encountered in the treatment of oral or intestinal bacteria, which on the laboratory scale and in fluid phase provide results in a dose-dependent manner.

The values recorded for plain liposomes, CHM anchored liposomes and SM-anchored liposomes are presented in Fig. 5. The % BGI (maximum at 90% MIC) offered by different formulations were 97.9% ± 6.1 for SM-anchored, 86.3 ± 4.5 for

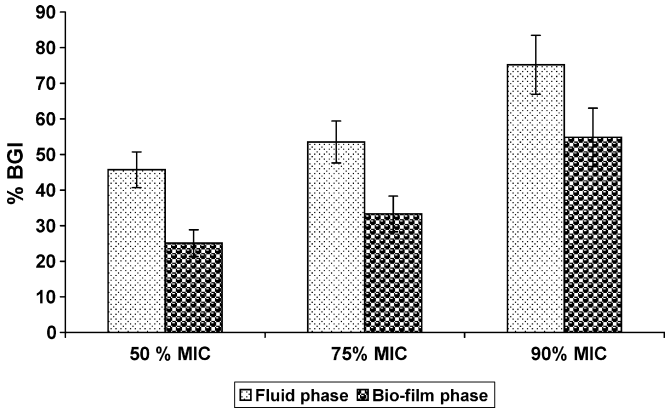



Fig. 4. Percent bio-film growth inhibition by metronidazole against fluid phase and bio-film phase bacteria. Values are expressed as mean ± S.D. ($n = 6$).

CHM anchored and 70.1 ± 3.8 for plain (unanchored) metronidazole loaded liposomes. Control versions (drug-free mannosylated liposomes) did not impart any significant growth inhibition effects ($P > 0.05$, data not shown).

The enhancement of anti-microbial activity when using mannosylated liposomes was most probably due to their ability to adsorb/adhere or fuse with the bacterial bio-film due to ligand mediated interactions and subsequent release of all or a part of anti-microbial agent in the vicinity of the target. These ligand–receptor interactions provided exceptional advantages over the free drug and drug loaded plain liposomes. Firstly, because the ligand-anchored vesicles were in direct proximity with the bio-film surface, the released drug would have a better probability for diffusion and access into the bio-film than the free drug in solution. Secondly, unlike the free drug, the adsorbed vesicles would not have been washed off the bio-film after the incubation period and were able to release their contents into the propinquity of bio-film surface during the regrowth assay. In all the investigated systems, SM-anchored liposomes demonstrated significantly better results compared to other formulations ($P < 0.05$).

The study revealed that plain liposomes though provide sustained effects as compared against plain drug, are less effective than mannosylated liposomes (statistically significant, $P < 0.05$).

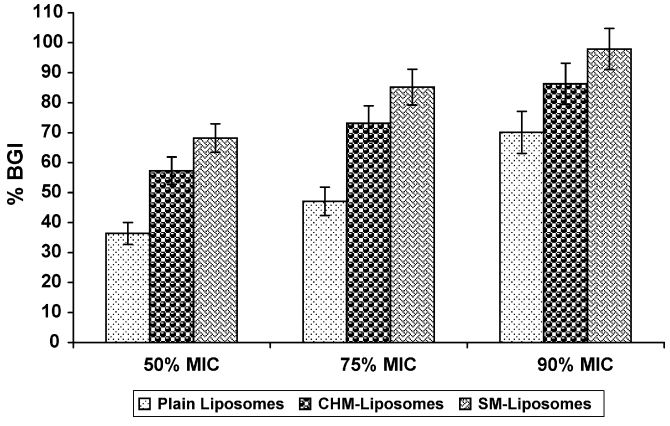



Fig. 5. Percent bio-film growth inhibition by different liposomal formulation. Values are expressed as mean ± S.D. ($n = 6$).

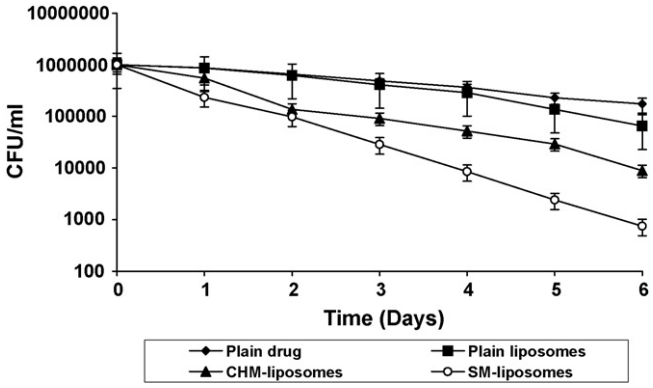



Fig. 6. Viable counts of bacteria after treatment with different formulations in rat pouch induced bio-film model. Values are expressed as mean ± S.D. ($n = 6$).

Cholesteryl mannan also however, provided better effect as imparted by plain vesicles (statistically significant, $P < 0.05$). Ligands like sialic acid, may recognize the counter ligands expressed on the bio-film environment thus paving the way for the ligand-mediated entry of the vesicles and subsequent release of the drug in the cellular interior providing a site-specific and targeted delivery to its maximum potential.

### 3.3.2. *In vivo CMC pouch infection model*

An in vitro model is although informative but does not give real bio-architecture of bio-films, which is composed of bacteria, bacterial exopolymers, various insoluble proteins and glycoproteins and blood cells. In an attempt to overcome these drawbacks, an in vivo *S. aureus* bio-film model in rat pouch was also developed in our study. The formulations were administered everyday for 5 days and the pouch exudates were tested for the reduction in the viable bacterial count (Fig. 6). Similar pattern of activity of different formulations as recorded in the in vitro microtitre plate model was observed in this case also. The plain drug was found to be least effective and could bring only <1 log 10 reduction in the viable bacterial count while SM anchored liposomes showed maximum effectiveness in in vivo conditions also. A >3 log 10 reduction was recorded when sialic acid conjugated mannan was used as a ligand. The fact that sialo-mannan anchored liposomes were superior in exhibiting better results indicate that attachment of the vesicles to the lectins expressed on bacterial bio-film would probably be a key factor in exhibiting significantly higher % BGI values both in vitro and in vivo ($P < 0.05$).

Various mechanistic points have been put forward in the literature for the synergistic action of liposomes with anti-microbial agents and/or antibiotics (Nacucchio et al., 1988; Hutchinson et al., 1989; Jones et al., 1994a,b, 1997; Robinson et al., 1998; Vyas et al., 2001; Jones, 2005). In our study, plain vesicles exhibited significantly better bio-film targeting index as compared against free drug ($P < 0.05$). The effects could be ascribed to the protection of metronidazole encapsulated in the vesicles from β-lactamases and exogenous enzymes and the local concentration of drug present per unit area per unit time is never higher to be inactivated by these factors. Change in the bacterial cell envelope permeability facilitating the fusion/diffusion of the drug across the bacterial envelope, followed by translocation of the contents, could also be cited as another possibility (Nacucchio et al., 1988).

Mannosylated vesicles produced significant levels of percentage BGI with respect to plain vesicles and free drug ($P < 0.05$), which could be due to one or more of the proposed mechanisms, which needs to be further substantiated. Jones and co-workers revealed that liposomes incorporating low levels of phosphatidylinositol (PI) would target to the bacterial bio-film (Jones et al., 1994a,b). The results from their study demonstrated that an interaction exists between the PI head groups and the surface polymers (probably via hydrogen bonding with monosaccharides) of the bacterial glycocalyx. These results prompted in the present work to select polysaccharide as one of the ligand and to check whether a similar targeting potential can be generated with the polyhydroxy –OH terminal groups available in the polysaccharides. However, to further extend the concept, we have realized the role of true ligands and anchored sialic acid (which serve as ligand for the lectin receptors expressed on bacterial bio-film) to this polysaccharide. The object was to assess the role of polysaccharides and sialo-polysaccharides for their targeting potential.

The multi- or polyvalency characteristics of the polysaccharides, i.e., binding to a target site through multiple interactions can be proposed to partially explain the enhanced activity of anchored module (Matrosovich, 1989). Ligands (anchored on the surface of liposomes) may simply arrest glycocalyx covering (glycoprotein and glycosphingolipids) by their multivalency characteristics and may cause peptization and sequestration of bio-films. These bio-film fusogenicity characteristics could be ascribed to the significant growth inhibition offered due to the selective targeting of the liberated anti-microbial agent at the site of infection.

## 4. Conclusion

In the light of these considerations, it can be suggested that the developed system(s) may offer potential in alleviating those drug bacterial resistance problems due to bio-film colonization of the causative organism. They could also be used clinically for bacterial infections in the conditions like plaque formation (periodontal pocket) or in arresting bio-film formation in the implanted devices.

However, the nature and type of bio-film may be associated with the same or different type of carbohydrate recognition domains and thus the uptake of mannosylated systems depends upon the complexity and heterogeneity of the bio-film skeleton and glycocalyx oligosaccharides. This fact should be considered while engineering the stable oral delivery systems for bio-film targeting.

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