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Preparation, characterization and *in vitro* antimicrobial activity of metronidazole bearing lectinized liposomes for intra-periodontal pocket delivery

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Liposomes constructed of egg phosphatidylcholine (EPC), cholesterol (Chol) and stearoylamine (SA) were coated with lectin (Concanavalin-A). These lectinized liposomes were found to retain the ligand binding activity of surface coated concanavalin A (Con-A) as demonstrated by bovine submaxillary mucin (BSM) binding assay. Moreover the ligand specificity of Con-A was maintained even after coating the liposome surface because the presence of competing sugar α-methyl mannoside, significantly inhibited the interaction of lectinized liposomes and BSM. The significance of divalent cations for these interactions was studied. The Con-A coating was found to be stable in simulated salivary fluids (SSF, pH 7.2) and under various pH conditions. *In vitro* targeting studies of lectinized liposomes with gram-negative bacilli (*Streptococcus mutans*) that harbor in the periodontal pocket (biofilm) demonstrated nearly 100% bacterial growth inhibition (% BGI). The antimicrobial effect was maintained for 360 min. The results were compared with metronidazole bearing plain (protein free/uncoated) liposomes and the free drug at the same dose levels. Mechanisms involved are also discussed. These observations suggest that liposomes coated with lectin (Con-A) were able to maintain the sugar affinity and specificity of the associated ligand and could be targeted to the surface 'glyco-calyx' of bacterial bio-film.

1. Introduction

Liposomes can be used as carrier systems for the presentation and transportation of various bioactives including antibodies, polypeptides, oligosaccharides (carbohydrates), viral proteins, fusogenic residues and lectins [1]. The ligands either of endogenous or exogenous sources confer specific avidity to the drug carrier or vector systems and lend them to selectively deliver the drug to the cell or group of cells equipped with particular receptor units [2].

The lectin-carbohydrate interaction constitutes the basis of carbohydrate mediated cellular events like cellular adhesion, differentiation and recognition processes. The possibilities of incorporation or immobilization of various site directing carbohydrate ligands on to the liposomal surface have led to the development of a wide range of delivery systems based on carbohydrate-lectin interactions [3]. Potential liposomal drug carriers based on carbohydrate mediated recognition are abound in the literature [4–8].

One of the most relevant techniques that exploit the carbohydrate-lectin recognition concept is the coating or coating of lectin to the liposomal surface to engineer a proteoliposome, which should retain the specificity and affinity of the immobilized lectin towards its affinity sugars. Lectinized liposomes have been used as a means of targeting to chicken erythrocytes and mouse spleen cells [9], HeLa cells [10], mouse fibroblasts [11] and various oral and skin associated bacteria [12].

Periodontal pocket disease is a collective term for a number of pathological conditions characterized by inflammation and degeneration of the gums (gingiva), supporting bones (alveolar bone), periodontal ligament and cementum. Bacteria develop in the periodontal pocket as a plaque (bio-film) and behave rather differently in their pharmacological and metabolical manifestations, thus making their eradication difficult [13]. Lectinized liposomes are reported to have considerable potential as a delivery system for the control of dental plaque and gingivitis [14]. In the present study lectin (Con-A) was coated on liposomal surface adapting a novel procedure. Its stability in the fluids of different osmotic status and pH was checked. The system developed was finally evaluated for its targeting potential *in vitro* against fluid phase *Streptococcus mutans.*

2. Investigations and results

Several antimicrobial drugs such as minocycline, spiramycin, tetracycline, chlorhexidine and doxycycline are known to be effective against periodontal bacterial infections [15]. Although many periodontopathogens are susceptible to the imidazole analogues, tetracyclines, penicillin, erythromycin, spiramycin, amoxycillin, clavulanic acid and clindamycin, at concentrations that can be achieved in body fluids, none could inhibit all bacteria currently implicated or suspected as aetiological agents in periodontal pathogenesis (reviewed in [16]). However, metronidazole remains the drug of choice in the treatment of periodontitis because of its wide spectrum of activity, low toxicity (LD_{50}) and high efficacy against gram negative bacteria ($MIC_{50} \sim 18$ μg/ml).

It has been recently realized that the bacteria develop as a sticky film of tangled polysaccharide fibre in the plaque and inflammatory periodontal disease. Not only these forms (bio-film) resist antimicrobial agents but they are also large enough to defeat the immune system. It is therefore pivotal to investigate ways to target antimicrobial agents to the bacteria (or in the vicinity of the bacteria). Among the various delivery systems directed against the intraperiodontal pocket flora, liposomal systems were found to be versatile in their drug disposition. The present study investigates lectin-carbohydrate (sugar) mediated delivery of metronidazole against the bacterial flora in the periodontal pocket. Lectin (Con-A) coated liposomes were investigated for their interaction with sugars expressed on cell surface glyco-calyx (a skeleton composed of glycoproteins, sphingolipids and glycolipids projecting terminal sugars to be recognized by lectins) of bacteria in periodontal pocket infections.

In the present study liposomes were prepared using the lipid film hydration technique and the drug (metronidazole) was loaded (0.20 μg/μg of lipid) from the aqueous

phase during the hydration of the lipid film deposited on the inner walls of the rotary flash evaporators. This was followed by separation of the unentrapped drug by centrifugation (100,000 g for 30 min) and trapped drug in the liposomal pellets was estimated using HPLC following their disruption using triton X-100. Drug loaded plain (uncoated) liposomes were surface appended with Con-A. Liposomes (stearoylamine based) were lectinized with Con-A at physiological pH (7.4). Dicetyl phosphates based liposomes also showed significant coating with Con-A at pH 6.0, but were not used in further studies as Con-A is reported to exhibit maximal polysaccharide specificity at pH 6.5 to 8.0 [17]. Moreover, Ca^{++} used in the ligand activity and specificity assay induced fusion in the case of negatively charged liposomes. However, it is obvious that an electrostatic and charge-induced interaction could work for the coating of liposomes with Con-A due to the zwitterionic characteristics of the latter. Surface charge of the liposomes prior to and after the coating of the lectin suggests charge-induced coating and quenching. It could be seen from Table 1 that the charge of liposome dispersion (before coating) contributed due to positively charged stearoylamine ($\sim +35.7$ mV) was reduced sequentially upon addition of the lectin (Con-A, which carry a negative charge above its isoelectric pH) from 0.001 to 0.01 w/w to total lipids. Further increasing the concentration of Con-A to a total lipid weight ratio of 0.1 : 1 w/w results in a negative Zeta potential ($\sim -5 \pm 2$ mV). This ratio signifies the binding of Con-A to the oppositely charged liposomes. The Zeta potential of -5 ± 2 mV recorded even after coating of Con-A could be attributed to the residual charge imparted by the excess Con-A to the dispersion. A further increase beyond this ratio contributes to a more negative Zeta potential of the dispersion. However, in our study the weight ratio of lectin to total lipid, 0.1 : 1, was taken to ensure coating of Con-A over positively charged liposomes constructed of steroylamine.

Unconjugated Con-A was separated from the lectinized liposomes using three cycles of centrifugation (100,000 g) of 30 min each, after which a plateau of Con-A concentration (estimated using Wang and Smith modified assay) was recorded. This would eliminate and delodge physisorbed material. Subsequent cycles did not result in any increase in the unconjugated (free) Con-A concentration, signifying not only the separation of the unconjugated Con-A, but also its resistance towards delodging under mechanical agitation.

Lectinized liposomes were characterized for vesicle size, shape, surface charge, vesicle size distribution and % entrapment. Drug loaded plain (uncoated) liposomes were found to be multi-lamellar and spherical in shape with vesicles ranging from 1.5 to 6.0 μm having a mean vesicular diameter of 2.9 ± 0.9 μm. The mean vesicle size of the drug loaded lectinized liposomes and drug loaded plain liposomes (average vesicle size 2.8 ± 1.2 μm) were comparable. However, the lectinized vesicles appear opaque and this may be accounted to protein coating, which resulted in a dual diffusion barrier on the liposomal surface. % Entrapment was recorded to be marginally decreased in the lectinized vesicles from an average of $27.1 \pm 0.8\%$ to $22.8 \pm 0.8\%$. The decrease could be attributed to the residual drug leakage from the vesicles during the incubation time lag employed for coating of lectin to the outer half of the liposomal bilayers. Lectinized liposomes with their *in vitro* characterization parameters are presented in Table 1.

Lectinized liposomes were investigated for the functional affinity and activity of the coated ligand after its immobilization on liposomal surface. The ligand related activity was evaluated and established by bovine-submaxillary mucin (BSM) interaction with lectinized liposomes. The addition of Ca^{++} in mM quantities to the reaction mixture is a prerequisite for the Con A-sugar recognition and is already established [18]. Ca^{++} activates metalloprotein lectins in carbohydrate binding assay. Usually 1 mM of Ca^{++} has been employed, but increased binding of immobilized lectin has been achieved using cation concentrations higher than the usual 1 mM and recommended for increase in sensitivity of the binding assay. This may be due to the fact that the cation content of commercial sources of lectin conjugates may be diminished and it is therefore not, sufficient to add 1 mM of Ca^{++} in lectin binding assay [18]. Similar results were obtained in our study on addition of increasing concentration of Ca^{++} (mM) to the reaction mixtures. The response with increasing concentrations of Ca^{++} was somewhat greater, with a nearly five-fold increase at 10 mM and leveling off at the same value (Table 2). No significant change was observed on further increasing the Ca^{++} (mM) concentration.

Fig. 1 compares the BSM interaction at 1 mM (A and C) and 10 mM (B and D) Ca^{++} concentration, and it could be seen that though the degree of interaction was increased on increasing the cation concentrations in reaction buffer, but the pattern remained the same. Fig. 1 represents the amount of BSM which interacted with the lectinized liposomes or plain (uncoated) liposomes (used as control in this case) at pH 7.4 (A and C). Interaction levels are presented with an incubation time of 60 min with the BSM. Experiments were carried out in the absence (for the study of *in vitro* activity) or in the presence of the specific sugar, α-methyl mannoside for Con-A (for the study of *in vitro* specificity). In the absence of α-methyl mannoside, lectinized liposomes showed (A and C) more than two-fold interaction with BSM as compared to the interaction with uncoated plain liposomes (used as control). % BSM interaction recorded by plain liposomes

Table 1: *In vitro* characterization of various lectinized and drug loaded plain liposomal formulations

Composition[a]	Lectin to total lipid ratio[b]	Vesicle size (Avg)[c]	% Entrapment[d]	Surfacial charge[e]
PC : Chol : SA	–	2.8 ± 1.2	27.1 ± 0.8	$+ (35.7 \pm 2.1)$
(PC : Chol : SA) : Con-A	0.001 : 1	2.9 ± 0.9	22.4 ± 0.6	$+ (14.9 \pm 0.9)$
(PC : Chol : SA) : Con-A	0.01 : 1	2.9 ± 0.9	22.4 ± 0.6	$+ (2.7 \pm 0.5)$
(PC : Chol : SA) : Con-A	0.1 : 1	2.9 ± 1.1	22.8 ± 0.8	$- (5.0 \pm 2.0)$
(PC : Chol : SA) : Con-A	1 : 1	3.2 ± 0.1	22.8 ± 0.8	$- (27.6 \pm 3.4)$

[a] The liposome compositions are based on a molar ratio of PC : Chol : SA (2 : 1 : 0.1)
[b] The ratio of lectin to total lipid is expressed on a weight basis
[c] Average vesicle size of previously screened and oversized (1.2 μm) population was obtained using phase contrast microscope coupled with calibrated eyepiece (n = 6)
[d] % Entrapment was analyzed using classical dialysis format (n = 6)
[e] Surface potential of the drug loaded lectinized liposomes at different weight ratio of lectin to total lipid (n = 3)

Table 2: *In vitro* ligand activity and specificity assay prior to and after adding the competing sugar

Formulations	% BSM interaction[b] at different mM Ca^{++}				
	1 mM	5 mM	10mM	15mM	20mM
Before adding competing sugar[a]					
Drug loaded plain (PC) liposomes	2.1 ± 0.1	2.1 ± 0.5	2.1 ± 0.1	2.1 ± 0.5	2.1 ± 0.3
Lectinized (PC) liposomes	7.6 ± 1.2	15.2 ± 1.2	22.1 ± 1.9	22.3 ± 2.1	22.7 ± 0.9
After adding competing sugar[a]					
Drug loaded plain (PC) liposomes	1.9 ± 0.6	1.9 ± 0.7	1.9 ± 0.1	1.9 ± 0.3	1.9 ± 0.2
Lectinized (PC) liposomes	1.6 ± 0.2	4.7 ± 0.5	8.9 ± 0.3	8.7 ± 0.9	8.7 ± 0.6

[a] Competing sugar used was ∝-methyl mannoside (MM)
[b] All experiments were run in triplicate

could be ascribed to non-specific adsorption of protein (BSM) over the vesicle surface. With no significant changes in the levels of BSM binding were recorded after the addition of α-methyl mannoside, it clearly signifies the nature of the non-specific adsorption. These results clearly indicate that surface coated lectin remained functionally active even after its coupling to lipid vesicles. Experiments performed in the presence of α-methyl mannoside are presented as bar diagram (B and D). In the case of plain liposomes (control) the results remained unvaried for liposome-mucin interaction either in the presence or in the absence of the specific sugar (for Con-A). In contrast, the interactions between the lectinized system and mucin decreased significantly on addition of competing sugar. The results clearly reveal that lectinized liposomes possess the sugar specificity that corresponds to the native lectin.

Lectinized liposomes were subjected to simulated salivary fluid composition of different pH values in order to check the physicochemical stability of the system against the osmotic status to be encountered in the periodontal pocket and also to long term storage to check its shelf stability in terms of Con-A latency. Since the coating of liposomes with Con A is the crucial point of the study, its association with liposomes is pivotal in establishing the stability profile of the developed system. The Con-A latency was calculated by estimating the Con-A concentration with respect to total lipid at the start and at the end of the experiment using Wang and Smith modified assay [19]. The lectin latency was calculated using the following formula:

$$\% \text{ Con-A latency} = \frac{[\text{Con} - \text{A}]/[\text{Phospholipid}]^1}{[\text{Con} - \text{A}]/[\text{Phospholipid}]^2}$$

[1] at the start of stability study
[2] at an appropriate incubation time

Fig. 2 shows the stability projection of lectinized liposomes in the presence of simulated salivary fluids of different pH values at 37 °C. The resistance of immobilized protein against different pH stresses was interestingly found substantial as a Con-A retention of almost 77.1 ± 7.5 % was recorded. An initial decline in % retention and hence in latency values could be ascribed to the delodging of adsorbed Con-A that would have been associated during the process of charge induced coating to the liposomal surface. Further it could be seen that on increasing the incubation time of liposomes with simulated fluid did not influence the latency profile significantly. The finding that no significant degradation occurred in dispersions in *in vitro* studies suggests that the protein cap offered better stabilization to the liposomal bilayer. It could be seen that the % cumulative drug released from lectinized liposomes during the stability experiments as measured from the dialysed fluids was suppressed as compared to drug loaded plain liposomes (data not shown). Even in this case, Con-A lectinized liposomes demonstrated an almost 1.5 times increase in drug latency (drug remaining associated with liposomes calculated from drug

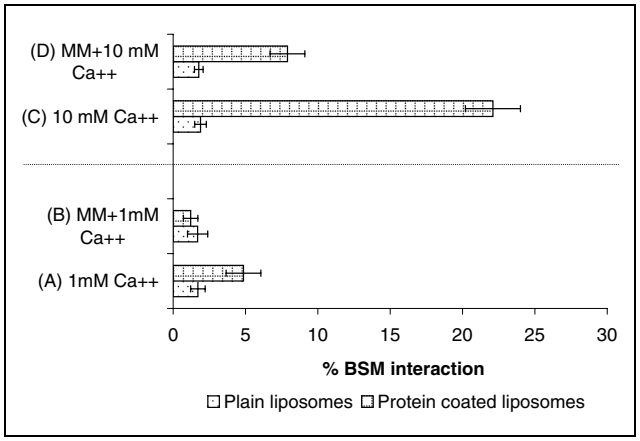

Fig. 1: BSM binding by lectinized liposomes and drug loaded plain liposomes prior to and after the challenge of the competing sugar, α-methyl mannoside. Figure A and C indicate the binding of lectinized and unlectinized liposomes before adding the competing sugar, α-methyl mannoside (MM) in the presence of 1 and 10 mM Ca^{++}. After the addition of MM (B and D), lectinized liposomes showed reduced binding to BSM. Drug loaded plain liposomes did not show any appreciable change in the binding affinity towards BSM in the presence or in the absence of competing sugar

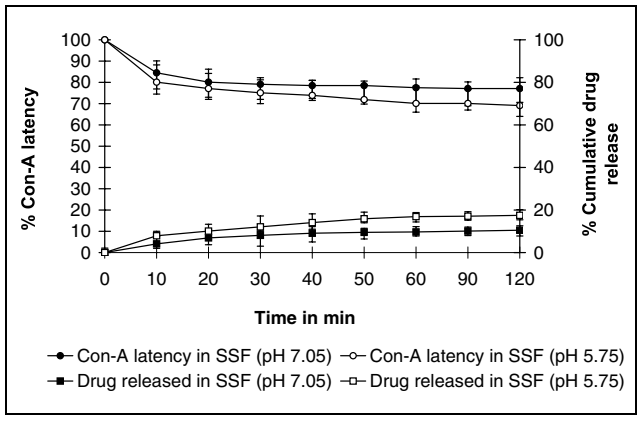

Fig. 2: Stability against pH stresses in different tonicity simulated salivary fluids (pH 5.75 and 7.2). Results are expressed in terms of Con-A latency values, i.e., Con-A remained associated with phospholipid, on primary Y-axis. % Drug released was also recorded at the same time periods from the dialysed fluid, and presented on secondary Y-axis

release data) against plain (protein free/uncoated) liposomes, showing their potential as stable modules against the physicochemical and mechanical challenges. Since the system is based upon ligand mediated localization of the entrapped drug, the suppression of drug efflux should not influence its targeting potential.

The antimicrobial effect of drug-loaded plain and lectinized liposomes was determined in terms of percentage bacterial growth inhibition (% BGI). % BGI was calculated as the ratio of optimal density (at 550 mm) of a given test mixture against that of tubes containing *S. mutans* alone and can be expressed as given below:

$$\% \text{ BGI} = \frac{[\text{OD of test organism - OD of test mixture}]}{[\text{OD of test organism at a given time}]} \times 100$$

The effects of different drug loaded and placebo formulations on bacterial growth were investigated. The formulations investigated for their antimicrobial activities include free metronidazole, control liposomes with buffer, drug loaded plain liposomes and drug loaded lectinized liposomes. Control liposomes included in the experiments were metronidazole free plain (control 1) and lectinized (control 2) liposomes in order to check any antimicrobial activity of the constitutive lipids or immobilized lectin.

The optical density procured by the addition of drug loaded plain and lectinized liposomes [final lipid concentration in culture media was 0.01 μm (7.2 μg/100 μl of the dispersion medium)] to the bacterial culture was not significantly different from that of tubes containing medium alone (without any bacterial culture). All the results of liposomal bacterial interactions are presented graphically (Fig. 3). Bacterial growth was significantly inhibited by drug loaded plain and lectinized systems to the levels of significance (Rank sum test). Further, the difference in growth inhibition when compared among the formulations was statistically significant ($P < 0.05$). Bacterial growth reductions by the developed systems were comparable to the effect of the free antimicrobial. These results demonstrate that the lectinized liposomes caused a significantly higher % BGI in the bacteria growth at an equivalent MIC value of encapsulated drug (MIC_{50}) than drug loaded plain liposomal formulations. In other words lectinized liposomes exhibited superior % antimicrobial activity compared to that of drug loaded plain liposomes or free drug as estimated from triplicates of representative experiments.

The tubes containing drug free liposomes (plain and lectinized) and bacterial culture were included in order to ascertain whether lipid peroxidation products or the coated lectin contributed to the bactericidal action exhibited by the prepared systems. Control tubes containing bacteria and plain liposomes (control 1) and lectinized liposomes (control 2) exhibited insignificant growth inhibition suggesting no antimicrobial activity of the constitutive lipids or immobilized lectin. The growth inhibition provided by the control tubes were 1.5 ± 0.9 % for control 1 and 4.1 ± 1.1% BGI for control 2, respectively. These values however reflect that they interact with the bacterial surface to varying degrees but do not possess their own anti-microbial properties.

Furthermore it was observed that when metronidazole (plain drug) was mixed with drug loaded plain and lectinized liposomes, antimicrobial activity (65.3 ± 4.5 % BGI) exceeded that of the free drug (27.1 ± 2.6%) significantly. This could be due to the fusion of vesiculogen with the bacteria in the fluid phase and simultaneous translocation of the metronidazole present in the fluid phase to the bacterial interiors.

The interaction of the drug loaded plain liposomes could be ascribed to the structural similarity of the liposomes with bio-membranes and diffusion of contents, whereas lectin-mediated adhesion to bacterial glyco-calyx could be instrumental in the case of lectinized liposomes. The lectin-appended system was proven to be the most effective in bringing the maximum growth inhibition (nearly 100 %) for 360 min. Statistical analysis of data revealed that there were significant differences in % BGI of drug loaded plain and lectinized modules at a 5 % level of significance ($P < 0.05$).

The level of entrapment of metronidazole within the drug loaded plain liposomes had an effect on % BGI values (Fig. 4). The higher the concentration of metronidazole in the aqueous cores of plain liposomes, the higher the trans bilayer concentration gradient and the greater the diffusion into the surrounding medium. However, the effect was pronounced in the case of short incubation time periods. Increasing the incubation times lowered the threshold metronidazole concentration at which growth inhibition was concentration-dependent. With longer incubation times, the entrapped contents had a longer time to leak out, so

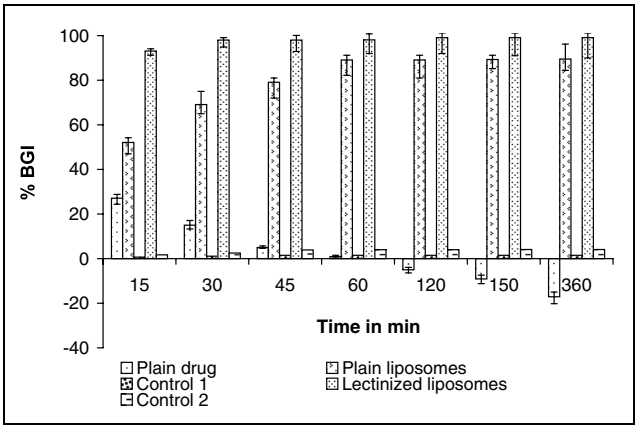

Fig. 3: % Growth inhibition achieved by drug loaded lectinized and plain liposomes at 0.5 MIC (~18 μg/ml) as compared against plain drug solution, calculated using spectrophotmetric method. Controls used were drug free plain (control 1) and lectinized liposomes (control 2). Different formulations were added to the tubes maintained aseptically throughout the experiment. Bacterial growth was monitored spectrophotometrically at 550 nm against blank (uninoculated broth) at different time intervals

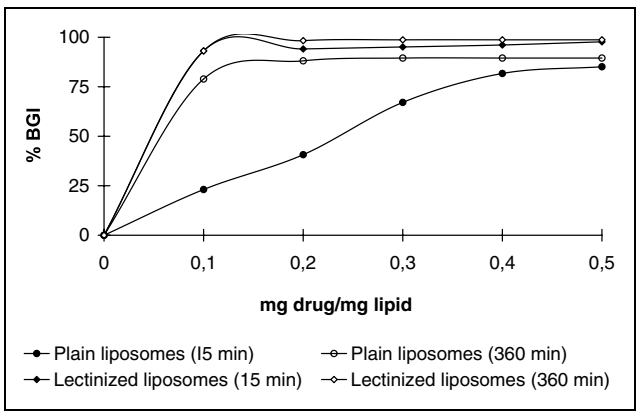

Fig. 4: Effect of the concentration of metronidazole in liposomes (drug loaded plain and lectinized) on inhibition of bacterial growth (% BHI). Various concentrations of metronidazole ranging from 0.1 mg/mg lipid to 0.5 mg/mg of lipids were used. Two sets of experiments were performed at short incubation (15 min) and long incubation (360 min) periods

the drug concentration at which vesicles with lower entrapped drug levels can cause comparable levels of growth inhibition. However, the lectin-coated liposomes were found to be independent of the concentration gradient or incubation times. This could be due to the fact that the drug targeting was predominantly result of the specificity of the Con-A towards the glucose- and mannose-residues of the bacterial surface glycocalyx. The lectin-carbohydrate interaction could lead to locus of defects in the membrane integrity and destabilization resulting into the burst release of the contents in the vicinity of the target cells.

3. Discussion

Assemblage of Con-A on the surface of liposomes lend the vesicles mechanically stable and offer them stability against external stimuli along with suppressed leakage of water soluble drugs. The latter has not been discussed frequently in the literature featuring proteoliposomes. Some of the workers however, ascertained in *in vitro* experiments that the permeability coefficients of proteoliposomes depend on the amount of surface immobilized proteins (an excess could lead to immunological consequences, either local or systemic) [20, 21]. This was suggested to be due to the dislocation introduced into the bilayer by the lipid molecules, which are keyed, to the protein, as a result creating free volume available for the passage of encapsulated contents. In our study, meanwhile the charge-induced deposition of Con-A to the liposomal surface excludes the use of anchors ensuring vesicle integrity towards membrane defects that are likely to arise in the vicinity of gel transition temperatures. Coating of Concanavalin A was successfully offered on steroylamine bearing liposomes (above the isoelectric point of lectin) and with dicetyl phosphate bearing liposomes (below isoelectric point of Con-A). Adsorptive coating of Con-A recorded for neutral liposomes (as evident from turbidometric analysis) was insignificant as compared to charge-induced coating of Con-A with charged bilayers, but its contribution cannot be ruled out. Since coating has been essentially based on electrostatic binding of ligands on to the surface, it may also involve to some extent conventional epitexial and paratexial phenomena of adsorption. Therefore, in majority this phenomenon is not a dispersion artifact or an unspecific adsorption of lectin to a lipid surface, rather, it can be concluded to be electrostatic in nature. Nevertheless, the system demonstrated noticeable stability against pH stresses, which apparently attributes to the immobilization of ligand and eventually offered deterrence. However, in view of its otherwise expected reversible electrostatic interaction, these results require a well-planned study protocol in order to establish mechanistic involved offering protection to electrostatic interactions under pH stresses. Our group took lectin-coated liposomes (lectinized liposomes) constituted with stearoylamine for the *in vitro* stability and bacterial interaction studies.

Lectins function as potent exogenous biological signal and can affect peripheral organs and bacteria associated with them. Their ability to resist the gut proteolysis and a high specific chemical reactivity with endogenous surface receptors of the endothelial cells of gut of both higher animals and lower organisms, make them suitable ligand molecules for the targeted drug delivery by oral/peroral route. The binding specificity of lectins for sugars [22] make them an interesting alternative for targeting liposomes to the glycocalyx of the membrane surface and other bio-surfaces [23]. Analogous to previous report, which describes that chemical modification of Con-A does not lead to any significant changes in lectin affinity towards saccharides [24], no change in the ligand affinity and activity of Con-A (immobilized on liposomal bilayer) was detected. Ligand activity was found to be intact as lectinized liposomes showed nearly two-fold interactions with bovine submaxillary mucin (BSM). The ligand affinity of the coated ligand remained functional as the competing sugar (α-methyl mannoside) significantly reduced the interaction between BSM and the lectinized liposomes. Similarly, lectin immobilized on liposomal surface was instrumental in providing structural integrity and bilayer stabilization against simulated salivary fluids containing mucin and ptyalin. These results suggest exceptional stability of the lectinized liposomes and may be attributed to the protein (lectin) coating on liposomal system.

The efficiency of targeting of lectinized liposomes to *S. mutans* suspension culture has been measured in terms of % BGI. The experiment was conducted with metronidazole concentrations to an equivalent dose of 50% of the MIC ($\sim$18 μg/ml) of the free drug in all formulations and control. Drug loaded lectinized liposomes followed by drug loaded plain liposomes were found superior in maintaining a higher value of % BGI over a prolonged period of time. It was gradually leveled off signifying the higher and sustained % growth inhibition values for the lectinized module. Drug loaded plain liposomal formulation has been found to provide a higher % BGI compared to plain drug. This could be attributed to the intrinsic protection of antimicrobials encapsulated in the liposomes from β-lactamases and exogenous enzymes. A 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 [25, 26] however this would be substantial at longer incubation time periods.

Lectinized liposomes produced significantly better % BGI values, which could be due to one or more of the proposed mechanisms. The ability of lectin to bind to a target site through multiple interactions (multivalency) can be proposed to partially explain the enhanced activity of Con-A coated liposomes [27]. It could be assumed that lectinized liposomes with affinity to the specific sugars or sugar projecting glyco-conjugates of oral and skin associated bacteria are instrumental in pursuing better results [14]. These findings are based on the fact that lectin and the complementary carbohydrates are located on the surfaces of opposing cells, which may of the same type or different cells [3]. Cells of bacterial bio-film may interact with lectin immobilized liposomes via a bridge formed by soluble glycoconjugates that bind to the immobilized lectins. Alternatively, the immobilized lectin may combine with carbohydrate of insoluble component of the extracellular matrix that promotes vesicular-cell adhesion. Con-A has a specificity for glucose and mannose residues [28], which constitute a part of surface polymer of the bacterial glyco-calyx. However the bacterial composition of the bio-film in the periodontal pocket may vary considerably, the sugars most commonly expressed on surface glyco-calyx are specific for concanavalin-A.

In the light of these considerations, it can be suggested that the developed system may offer potential in alleviating drug bacterial resistance and multi drug resistance problems, and could be used clinically for periodontal pocket bacterial diseases and biofilm infections associated with indwelling medical devices.

4. Experimental

4.1. Materials

Egg phosphatidylcholine (EPC) was purchased from Sigma (St. Louis, MO, USA) and used as supplied. Cholesterol (Chol), dicetyl phosphate (DCP), stearoylamine (SA) and bovine submaxillary mucin (BSM) were from Fluca chemicals, Switz. Concanavalin A (Con-A) from C*oncanavalis easoniformis* was from Sigma (St. Louis, MO, USA). All other chemicals used were of analytical grade, unless otherwise mentioned and were obtained from Qualigens (Glaxo India Ltd.). *Streptococcus mutans* bacteria (MTCC 7447) were obtained from the Institute of Microbial Technology (IMT, Chandigarh, India) as bacterial strips. The metronidazole MIC was determined by the dilution susceptibility test as described by Baker et al. [29]. Metronidazole MIC_{50} for *Streptococcus mutans* was recorded to be $\approx$18 μg/ml as estimated from triplicates of representative experiments. The metronidazole dose is 50% of the MIC of free drug in drug loaded plain and lectinized liposomes as well as in plain drug formulation.

4.2. Preparation of vesicles by lipid film hydration technique

Plain (uncoated/protein free) liposomes were prepared by first removing the solvent using a rotary flash evaporator from a 250 μmol mixture of EPC, Chol and charge imparting constitutive lipids to form a thin film on the inner wall of a round bottom flask. The casted film was hydrated using mixed phosphate buffer of different pH values and the contents of the flask (vesicular dispersion) vortexed for about an hour to get the lipids off from the wall of the flask of flash evaporator into the dispersion. Metronidazole was dissolved in the aqueous phase (phosphate buffer) and incorporated at a concentration level that corresponds to 1 : 5 weight ratio of total lipid. These drug loaded plain liposomes were centrifuged thrice (100,000 g) for 60 min to remove any unentrapped metronidazole. The liposomal population were harvested and screened for vesicle sizes and those above 1 μm (as retained oversized on Acrodiscs, Gelman, 1.2 μm) were taken for coating and subsequent stability protocols.

4.3. Protein coating of the vesicles

Coating of liposomes with lectin (Con-A) was accomplished by charge induced interaction of the protein over the oppositely charged lipid bilayer. The zwitterionic behavior of concanavalin A was exploited. Lectin acquires a different charge status above and below and in the vicinity of its isoelectric point [17]. To the whole vesicular dispersion obtained (total lipid $\cong 7.2 \times 10^{-2}$ μg ml^{-3}) 100 μg of Con-A dissolved in minimum volume (just sufficient to dissolve) of mixed phosphate buffers of varied pH was added. The pH of the dispersion was adjusted to pH 7.4 in the case of stearoylamine based formulations and to pH 6.0 in the case of dicetyl phosphate based formulations using mixed phosphate buffers. The dispersions obtained were kept incubated for an optimized period of 6 h. Charge-induced coating of Con-A over liposomal surface was further assessed using a Nephelometer (York, India), which recorded the changes in turbidity prior and after the addition of Con A. However to check the same quantitatively, the surface potential of the vesicles at different time intervals was recorded using a Zeta-potentiometer (Aplex 35, France) and the Con-A to total lipid weight ratio was optimized. The surface charge was calculated using Smoluchowski's equation from the mobility of liposomal dispersion in PBS (pH 7.4, 0.001 M) using the Zeta-potentiometer [30].
Unconjugated Con-A from liposome conjugated Con-A was separated by centrifugation at 100,000 g in 30 min and experiment was repeated to 7 cycles. Aliquots of the clear supernatants obtained from centrifugation during the removal of the free lectin were taken and estimated using the Wang and Smith modified Lowry assay [19]. The quantity of lectin bound to the vesicles was calculated as the difference between the initially added lectin and the lectin, which was recovered after centrifugation.

4.4. In vitro characterization

Vesicle characterization for size and shape was performed using transmission electron microscopy (TEM). Phosphotungstic acid (1%) was used as a negative stain (JEM 1200, EX 11, JEOL, JAPAN). Carbon lectinized samples were treated with albumin to render the surface hydrophilic, 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 through a 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 the average vesicle size was calculated.
The % entrapment was determined and expressed as the percentage of added drug incorporated in the vesicles. The yield was referred to as the ratio of the experimentally measured amount of the drug in the dispersion and the theoretical amount used. Predialysed vesicular dispersions were centrifuged at 100,000 g for 60 min. Pellets thus obtained were resuspended in 0.01M PBS (pH 7.4) and the process repeated 3 times. Vesicles were lysed by adding 1.0 ml of 0.1% v/v Triton X-100, centrifuged and the liberated contents were analyzed for metronidazole on a silica gel 60 F HPLC column at a detection wavelength of 254 nm [31]. Liposomes were assessed in terms of total lipid phosphorus. The concentration of phospholipids was determined by measuring inorganic phosphate after acid hydrolysis at 180 °C in 70% $HClO_4$ [32]. % Entrapment was expressed as % entrapment/mg of lipid.

4.5. In vitro ligand affinity and activity studies

The activity of liposome coated Con-A towards exogenously provided bovine submaxillary gland mucin (BSM) and the affinity towards competing sugar were studied [33]. BSM is a glycoprotein consisting of six different sugars namely, *N*-acetylglucosamine (168.0 μg/mg BSM); *N*-acetylgalactosamine (69.2 μg/mg BSM); galactose (15.2 μg/mg BSM); mannose (2.07 μg/mg BSM); fucose (9.53 μg/mg BSM) and sialic acid (16.9 μg/mg BSM). Different concentrations of Ca^{++} were added to the reaction mixture to activate binding specificity of immobilized lectin with carbohydrates [34]. The *in vitro* biological activity of the lectinized system was determined by mixing 1.0 ml of BSM solution in phosphate buffer (0.5 mg/ml) with the same volume of dispersion of the lectinized liposomes in phosphate buffer (50 μg of liposome surface bound lectin per ml). After incubation for 60 min, the samples were centrifuged for 30 min at 100,000 g, aliquots (20 μl) of supernatant were taken, and analysed for BSM using HPLC analysis [33]. Assessing the difference between the total (reference system) and the residual BSM in the clear supernatant, the amount of interacted BSM was calculated. The reference system consisted of the same amount of BSM as in the sample (250 μg/ml in saline buffer), centrifuged as described earlier.
The ligand specificity studies were performed using α-methyl mannoside (50 mM) as a competing sugar in the BSM bulk solutions. % BSM interaction levels were recorded with the lectinized liposomes following the same protocol as used for the ligand activity studies.

4.6. In vitro stability studies

It is apparent to understand that although the system is intended for periodontal pocket (pH 7.0–7.4) administration, the studies concerning its fate at different pH and in fluids of different osmotic status, are significant. The stability of the lectinized liposomes was assessed in simulated salivary fluid (SSF) of different osmotic status likely to be encountered in the salivary fluid of the normal and pathogenic states. Dispersion of lectinized liposomes were dialyzed against salivary fluids of different pH values, and then again dialyzed back against PBS (pH 7.4). Simulated salivary fluids of pH 5.75 and 7.05 with osmolarity hypotonic to plasma were used. These SSFs were enriched with 5,000 units of ptylin per 10 ml, 2 g/l yeast extract, 5 g/l protease peptone, 2.5 g/l hog gastric mucin, 0.35 g/l sodium chloride, 0.2 g/l calcium chloride and 0.2 g/l potassium chloride. Stability studies were carried out using the equilibrium dialysis technique with various buffers as dialyzing medium under gentle magnetic stirring at 37 ± 1 °C for 12 h, in order to decrease the time of experiment. At different incubation times, aliquots of dispersion were removed and centrifuged (40,000 g for 30 min) to remove the free or delodged Con-A. Then the Con-A latency per mole lipid was calculated and expressed as the percentage of the same remaining associated with liposomes. The Con-A was estimated using procedures mentioned elsewhere [29]. The amount of drug released in the dispersion during the stability studies was also estimated and cumulative % drug released was plotted as a function of time.

4.7. In vitro targeting elucidation

In vitro targeting studies were performed on axenically grown broth culture of S*treptococcus mutans*. % Bacterial growth inhibition (% BGI) was the parameter studied. *Streptococcus mutans* bacterial strips were used to inoculate agar plates prepared from Brain heart infusion, BHI (3.7 g) in double distilled water (100 ml) to which was added bacteriological agar (1.5 g). The 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 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 lectinized 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 re-suspended in sterile PBS. The centrifugation and re-dispersion were repeated three times and the bacterial cell concentration appropriately adjusted by dilution with phosphate buffer for measuring the absorbance at 550 nm.
The antimicrobial effects of metronidazole bearing plain and lectinized liposomes were investigated on *Streptococcus mutans*, an organism predominantly present in periodontal pocket infections. Brain heat infusion broth (4.5 ml) was inoculated with 2.5×10^8 colony-forming units (CFU) of *S. mutans*, obtained from an early stationary phase culture. Different systems (drug loaded plain and lectinized liposomes, drug free or control plain and lectinized liposomes and plain drug) were added to the tubes to a final volume of 5.0 ml per sterile tube. The tubes were incubated under constant agitation at 37 ± 1 °C; bacterial growth was monitored spectro-

photometrically at 550 nm against blank (un-inoculated broth) at different time intervals. Every experiment was conducted in triplicate maintaining aseptic conditions. The drug alone in a final concentration that corresponds to 50% of MIC was added in the sterile tubes. Similarly in another set of experiment an equivalent amount of drug (MIC_{50}) was added from the metronidazole bearing plain and lectinized liposomes. After incubation of a bacteria-vesicle mixture for the stated period of time, growth inhibition was measured periodically for different formulations. The growth was recorded by measuring optical density (550 nm) of the dispersion mix using a Shimadzu 1601A UV/VIS DB spectrophotometer [26].

4.8. Statistical analysis

The anti-microbial activity of metronidazole loaded plain and lectinized liposomes against *S. mutans* in fluid phase was compared with that of plain drug and control formulations (placebo liposome formulations, plain and coated, without drug) using a rank sum test. The significance was evaluated at 5% probability level ($P < 0.05$ denoting significance).

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