Engineered vesicular constructs for biofilm targeting

SUMMARY AND CONCLUSION
The fields of pharmaceutical and biomedical sciences have been revolutionized by the fact that most bacteria in bio-environment aggregate as biofilms. This is a form in which they behave very differently as compared to free floating (fluid phase, planktonic) bacteria growing in laboratory cultures, upon which most, if not all, experiments have been performed to date. Biofilms are considered as microbe based-ecosystem attached to surface of biomaterials and biological sites. These single and/or multiple bacterial species efficiently co-ordinate and co-operate as biofilms and protect themselves against environmental stresses, facilitate better nutrient uptake for survival and impose resistance to conventionally applied antibacterial agents (Costeron et al., 1993; Potera, 1996; Sihorkar and Vyas, 2001).

Bacterial biofilms harbor and develop on or within indwelling medical devices, e.g., catheters, mechanical heart valves, pacemakers, and intrauterine devices (Costerton, 1999). However, plaque is the most natural biofilm that develop on human teeth and serves as etiological factor for caries and periodontal diseases (Vyas et al., 2000). Apart from plaque, biofilms are recently reported to accumulate on infectious biological sites as found in gastric ulcers, gastroenteritis and vaginitis.

Bacterial biofilms develop on these sites irreversibly adhere to the submerged surface and produce extracellular polymers. This extracellular polymeric substance (EPS) facilitates adhesion and provides a heterogeneous structural matrix. Biofilm-associated microorganisms thus behave differently from planktonic (exist in suspension culture) organisms with respect to growth rates and ability to resist antibacterial treatments (transport and physiological resistance) and therefore lead to problems like multi-drug resistance (Stewart, 1996).

The problem of drug resistance against biofilm infections needs a critical evaluation as to whether the therapeutic agents themselves are not effective or their effective presentation and localization to the biofilm environment is difficult with the available conventional means. After realization of the biofilm existence and moreover their recalcitrance towards conventionally adopted strategies, research has been shifted towards novel drug delivery and targeting approaches.
Novel drug delivery concept in general and vesicular carrier (liposome and niosome) technology in particular has provided the options and opportunities for designing and practicing the site specific and targeted drugs therapy. They offer flexibility to be engineered or tailored according to needs with exquisite levels of specificity and targetability. The structural versatility of vesicular systems in terms of vesicle size, shape, surface morphology, composition, surface charge and bilayer fluidity; the ability to incorporate a wide range of drugs; or to carry cell-specific ligands are pivotal to render them clinically and therapeutically beneficial and clinically adoptable (Gregoriadis and Florence, 1994). The topic selected for research work deals with the development and characterization of vesicular constructs appended with suitable site directing ligands for biofilm targeting. The approaches of using ligand anchored drug delivery systems for targeting may prove useful in delivering biocides into biofilms of oral and visceral species.

The effective drug delivery module to target the encapsulated drug to various biological sites where bacterial infections have been established as biofilms, like periodontal pocket, gastric mucosa and intestinal tract, is yet to be discovered. Though the vesicular constructs are well documented for targeted drug delivery, their adoptability for oral administration as drug carriers remain an unresolved issue around which at large the drug delivery inventions revolve. We attempted to prepare physicochemical stable vesicular systems that could deliver the entrapped bioactive to gastrointestinal tract located target site (here biofilms). In light of stated aim, the present study was performed through in vitro, ex vivo and in vivo experimentation using developed models. The efficacy of the ligand anchored vesicular systems to target surface determinants (carbohydrates and lectins) present on the bacterial biofilms was also tested.

In the present study, liposomes and niosomes were prepared and coated with various polysaccharides and lectin (protein) ligands, namely, cholesteroyl pullulan (CHPu), sialo-pullulan (SCHPu) and Concanavalin-A (Con-A). These vesicles were studied for stability in bioenvironment and their selectivity in targeting and efficiency in delivery of loaded drug to the biofilm. Staphylococcus aureus was used as a model
biofilm, as it is the causative bacteria of gastrointestinal (gastroenteritis), skin (implant/catheter-associated infections) and mucosal surface-associated infections.

The drug selected for the entrapment in the proposed systems was metronidazole, which is a broad-spectrum anti-bacterial agent and is proved to be effective against oral, vaginal, topical and intracellular infections (The Extra Pharmacopoeia, 1993; The Merck Index, 1996). A spectrophotometric method was used for the analysis of metronidazole for in vitro and in vivo studies. The calibration curves were prepared in buffer solutions of different pH values. The absorption maximum recorded were 277 nm, 277 nm, 279 nm, 320 nm and 320 nm in buffer solutions of pH 1.2, pH 2.2, pH 4.0, pH 6.8 and pH 7.4, respectively. This is in accordance with that reported in the literature (Sethi, 1997; Indian Pharmacopoeia, 1996; British Pharmacopoeia, 1998). The calibration curves were linearly regressed. Correlation coefficient (r) was found to be 0.997838, 0.998271, 0.998986, 0.99638 and 0.998116 respectively for calibration curves in pH 1.2, pH 2.2, pH 4.0, pH 6.8 and pH 7.4 respectively.

Similarly, calibration curves of phosphatidylcholine, Concanavalin-A and pullulan were prepared and these curves show good linearity. The values of correlation coefficients (r) were found to be 0.9992, 0.9966 and 0.9990 for phosphatidylcholine, Concanavalin-A and pullulan respectively.

8.1. PREPARATION AND OPTIMIZATION OF PLAIN AND LIGAND COATED VESICLES

Liposomes and niosomes were prepared by thin film hydration method (New, 1989) using different lipid constructs. The ratio of constitutive lipids was optimized for both liposomes and niosomes. Liposomes were prepared using either phosphatidylcholine (PC) or distearoylphosphatidylcholine (DSPC) along with phosphatidylethanolamine (PE) with or without charge imparting lipid. Niosomes were prepared using either SPAN 60 or SPAN 85 along with PE with or without charge imparting lipid. The drug, metronidazole was added in the hydration stage at different drug to total lipid weight ratio. Similarly, 6-carboxyfluorescein was also added in some formulations for the fluorescence microscopy studies in a weight ratio of 1:25.
The ratio of charge impartsing lipid (stearylamine) with total lipid was optimized based upon the vesicle integrity, shape and % encapsulation efficiency (%EE). It was seen that the % encapsulation was increased when the weight ratio of SA: total lipid was increased from 0.001:1, 0.005:1, 0.01:1, 0.05:1 and 0.1:1. However, no significant increase in % encapsulation was recorded after 0.05:1 ratio of SA to total lipids. This weight ratio (0.05:1) was taken as the optimized ratio in regard to amount of charge imparting lipid, stearylamine (SA) in the formulations. It can be summarized here that higher % encapsulation recorded could be ascribed to an increase in the inter-lamellae distance and entrapped aqueous volume of vesicles resulted due to charge induced repulsion amongst the bilayers.

Similarly, the ratio of PE in the formulation was optimized considering the membrane integrity and %EE of the formulations as a measure. Different weight ratios of PE to total lipid were selected for the optimization ranging from 0.001:1, 0.005:1, 0.01:1, 0.05:1, 0.1:1, 0.5:1 and 1:1. It was observed that beyond 0.1 (PE) to 1.0 (total lipid), some irregulars shaped structures were formed. When, PE to total lipid ratio was raised to 1:1 weight ratio, the vesicles were off spherical in shape and distorted in appearance. This can be attributed to the reported instability of the PE because of its rapid phase transition to non-bilyer (hexagonal phase II) structures (Litzinger and Huang, 1992). Moreover, the % encapsulation was also significantly reduced beyond this ratio due probably to increased defects in the membrane structure of the vesicles. However, relatively stable vesicles were formed when the weight ratio of PE to total lipid was kept below 0.1:1. This weight ratio (0.1:1) was taken as the optimized ratio for the amount of phosphatidylethanolamine (PE) invariably in all formulations.

With the optimum SA and PE ratio, the compositions of the remaining lipid fractions (PC or DSPC/SPAN and Chol) were varied and various formulations were prepared. The total lipid fractions were changed and different weight ratio of PC (or DSPC) and SPAN 60 (or SPAN 85) along with cholesterol were tried ranging from 85:5, 80:10, 75:15, 70:20, 65:25, 60:30, 55:35, 50:40 and 45:45. Accordingly, the formulation codes designated were PV1, PV2, PV3, PV4, PV5, PV6, PV7, PV8 and PV9 (for PC based liposomes) and DSPV1, DSPV2, DSPV3, DSPV4, DSPV5, DSPV6, DSPV7,
DSPV8 and DSPV9 (for DSPC based liposomes). Similarly, the formulation codes for niosomes were NV1, NV2, NV3, NV4, NV5, NV6, NV7, NV8 and NV9 (for SPAN 60 based niosomes) and NV’1, NV’2, NV’3, NV’4, NV’5, NV’6, NV’7, NV’8 and NV’9 (for SPAN 85 based niosomes).

These liposomes and niosomes were characterized for shape, size and % EE. The shape of the prepared vesicles was observed under a phase contrast optical microscope and was found to be multilamellar irrespective of the lipid composition and the type of the vesicles formed. In case of uncoated liposomes, formulations PV1, PV2, PV3, PV4, PV5, PV6, PV7, PV8, PV9, DSPV1, DSPV2, DSPV3, DSPV4, DSPV5, DSPV6, DSPV7, DSPV8 and DSPV9 recorded mean vesicle diameter of 2.31±0.23 μ, 3.05±0.13 μ, 3.76±0.21 μ, 3.87±0.16 μ, 3.85±0.19 μ, 3.12±0.45 μ, 3.08±0.23 μ, 3.56±0.56 μ, 3.91±0.56 μ, 3.12±0.35 μ, 3.41±0.51 μ, 3.65±0.62 μ, 3.21±0.35 μ, 3.76±0.31 μ, 3.87±0.45 μ, 3.69±0.24 μ, 3.15±0.36 μ and 3.91±0.15 μ respectively. In case of uncoated niosomes, formulations NV1, NV2, NV3, NV4, NV5, NV6, NV7, NV8, NV’1, NV’2, NV’3, NV’4, NV’5, NV’6, NV’7 and NV’8 recorded mean vesicle size of 1.23±0.33 μ, 1.95±0.43 μ, 2.35±0.23 μ, 2.64±0.25 μ, 2.85±0.41 μ, 2.53±0.15 μ, 2.67±0.31 μ, 2.88±0.25 μ, 3.12±0.56 μ, 1.06±0.15 μ, 1.41±0.45 μ, 1.62±0.56 μ, 1.53±0.25 μ, 1.89±0.34 μ, 1.79±0.15 μ, 2.24±0.53 μ, 2.63±0.15 μ and 2.56±0.41 μ respectively.

% Encapsulation of prepared uncoated vesicles were determined by first disrupting the vesicles using 1.0 ml of n-propanol: water (2:1 v/v), and then centrifuging and measuring the liberated contents at a detection wavelength of 320 nm. In case of uncoated liposomes, formulations PV1, PV2, PV3, PV4, PV5, PV6, PV7, PV8, PV9, DSPV1, DSPV2, DSPV3, DSPV4, DSPV5, DSPV6, DSPV7, DSPV8 and DSPV9 recorded %EE of 21.8±3.14 μ, 23.8±1.98 μ, 29.1±2.68 μ, 33.5±2.76 μ, 34.1±4.05 μ, 33.1±4.12 μ, 36.7±3.45 μ, 27.8±2.64 μ, 26.6±3.44 μ, 23.8±3.44 μ, 24.9±2.49 μ, 30.8±3.02 μ, 36.1±4.02 μ, 37.1±2.87 μ, 38.9±3.45 μ, 34.9±4.14 μ, 29.9±2.65 μ, and 27.9±3.44 μ, respectively. In case of uncoated niosomes, formulations NV1, NV2, NV3, NV4, NV5, NV6, NV7, NV8, NV9, NV’1, NV’2, NV’3, NV’4, NV’5, NV’6, NV’7, NV’8 and NV’9 recorded %EE of 22.8±2.85 μ, 24.1±2.76 μ, 26.5±2.87 μ, 28.8±2.45 μ, 26.7±4.12 μ, 34.5±2.55 μ, 33.4±3.15 μ, 38.5±2.32 μ, 35.3±2.08 μ, 16.9±1.43 μ,
18.2±2.76μ, 17.1±3.15μ, 19.5±1.97μ, 24.5±2.34μ, 27.1±4.09 μ, 29.7±1.34μ, 30.9±3.15μ and 31.4±2.81μ respectively.

The optimized in regard to % EE and formulations with comparative values (PV4, PV5, PV6, PV7, DSPV4, DSPV5, DSPV6 and DSPV7 for liposomes; and NV6, NV7, NV8, NV9, NV'7, NV'8 and NV'9 for niosomes) were selected for extrusion.

Selected multimellar vesicles were extruded 10 times through two-stacked polycarbonate filters of 0.4 μ pore diameter at a pressure of 100 psi using an extruder. Prior to the extrusion through the final pore size (0.4 μ), MLV dispersions were subjected to pre-filtration through a large pore size filter (1.0μ-2.0μ). This method helps prevent the membrane from fouling and improves the homogeneity of the size distribution of the final dispersion. Extrusion was carried out above the phase transitions (Tc) of the lipid (37±1°C for PC, 55±1°C for DSPC, 50±1°C for SPAN). Attempts to extrude below the Tc were proved unsuccessful as the membrane has a tendency to foul with rigid bilayers, which could not pass through the pores. Liposomes and niosomes thus formed were designated with the prefix VET added in to their formulation codes.

Vesicles prepared using extrusion technique (VETs) were subjected to gel-exclusion chromatography using Sephadex G-50 column to remove unentrapped metronidazole. These VETs were then further subjected to various evaluation parameters like size, shape, polydispersity and the %EE. The shape of the prepared vesicles was observed using transmission electron microscopy and was found to be unilamellar or plurilamellar irrespective of the lipid composition and the type of the vesicles formed. In case of uncoated liposomes, formulations VETPV4, VETPV5, VETPV6, VETPV7, VETDSPV4, VETDSPV5, VETDSPV6, VETDSPV7 after extrusion recorded mean vesicle size of 405±19 nm, 395±12 nm, 423±15 nm, 415±11 nm, 445±19 nm, 512±13 nm, 412.7±21 nm and 427±19 nm respectively. In case of uncoated niosomes, formulations VETNV6, VETNV7, VETNV8, VETNV9, VETNV’7, VETNV’8 and VETNV’9 after extrusion recorded mean vesicle size of 456±12 nm, 415±31 nm, 432±15 nm, 398±19 nm, 406±18 nm, 412±11 nm and 523±15 nm respectively.
%EE for liposomal VETPV4, VETPV5, VETPV6, VETPV7, VETDSPV4, VETDSPV5, VETDSPV6 and VETDSPV7 were found to be 28.9±3.4, 30.1±5.2, 28.4±6.1, 31.1±3.7, 32.4±2.9, 33.1±5.7, 36.6±7.8 and 32.7±5.9 respectively. %EE for niosomal formulations, VETNV6, VETNV7, VETNV8, VETNV9, VETNV'7, VETNV'8 and VETNV'9 were recorded to be 29.9±2.3, 31.1±6.8, 37.6±4.1, 33.1±1.9, 26.7±2.9, 27.2±6.3 and 28.8±4.8 respectively. Based upon the polydispersity and %EE, formulations VETDSPV6 (for liposomes) and VETNV8 (for niosomes) were selected for ligand anchoring studies.

VETDSPV6, with a lipid ratio of 12:6:2:1 (DSPC:Chol:PE:SA) provides highest %EE amongst the formulations tested and this may be accounted to the charge imparting lipid, higher phase transition nature of the DSPC as compared against PC and optimal concentration of cholesterol in the formulation, which rigidize the fluid bilayered structure and hence might have contributed to the higher %EE values determinative through trapping capability.

VETNV8, with a lipid ratio of 10:8:2:1 (SPAN60:Chol:PE:SA) provided highest %EE amongst the formulations tested. An interesting observation was recorded in which the %EE of all SPAN 60-based formulations were higher than those achieved in case of SPAN 85-based formulations. SPAN 60 (sorbitan monostearate) and SPAN 85 (sorbitan trioleate) have the same head group whilst SPAN 85 has an unsaturated alkyl chain. De Gier and co-workers (1968) demonstrated that the introduction of double bonds into the paraffin chains causes a marked enhancement in the permeability in liposomes. Same explanation may possibly hold relevant to explain the lower %EE recorded in the case of SPAN 85-based niosomes. Alternatively it could be attributed to the higher phase transition temperature of the SPAN 60 compared to SPAN 85. As for vesicle size increasing hydrophobicity of the surfactant monomer led to the small sized vesicles (HLB for SPAN 60 is 4.7 and SPAN 85 is 4.3), a result which might be anticipated since surface free energy decreases with increasing hydrophobicity (Wan and Lee, 1974).

Optimized liposomal VETs (VETDSPV6, renamed as PV) and niosomal VETs (VETNV8 renamed as NV) were subjected to anchoring of ligands, namely cholesterolyl
pullulan (CHPu), sialo-pullulan (SCHPu) and Concanavalin-A (Con-A). These coated vesicles were characterized for vesicle shape, size distribution, %EE, release rate and ligand activity and site specificity studies.

8.2. CHARACTERIZATION OF PLAIN AND LIGAND COATED VESICLES

8.2.1. Characterization of polysaccharide coated vesicles

The aim of anchoring the ligand was to provide stability to vesicular system and targeting these stable carriers to site of action, i.e. biofilms. The effect of polysaccharide coating on liposomal and niosomal VETS were assessed in in vitro studies and overall performance before and after the coating was compared. The Chol-modified pullulan derivatives (also termed as hydrophobized pullulan or hydrophobized polysaccharides) were chosen, namely, cholesteryl pullulan (CHPu) and sialo-pullulan (SCHPu).

The polysaccharide ligand chosen in the present study is a linear α-glucan (Pullulan) produced by yeast like fungus Pullunaria pullulans. Efforts of coating pullulan over liposomes via adsorption were failed, as it is easily desorbed on dilution or mechanical agitation (Iwamoto and Sunamoto, 1982). In our study, we coated liposomes and niosomes with Chol-modified pullulan via hydrophobic interaction and affecting insertion in to the bilayered membrane (Hammerling & Westpal, 1967; Sato & Sunamoto 1992). Pullulan as a Chol-pullulan derivative (CHPu) gets inserted/inter-digitized in to the bilayer as if being part of it. The derivatization product was confirmed using IR and 1H-NMR analysis, which suggests the presence of an ester bond between pullulan and hydrophobic moieties. These compounds were used for the synthesis of sialo-pullulan (SCHPu) using procedure reported by Kijima et al., 1982.

Both CHPu and SCHPu were added to optimized VET formulations followed by incubation. It could be proposed that the hydrophobic anchors interact with the outer half of the bilayer orienting and projecting hydrophilic portion towards the aqueous bulk. In this way a two dimensional network of polysaccharide is structured around the vesicle membranes. Different weight ratios were selected to coat CHPu and SCHPu with respect
to total lipids (1:10, 1:6, 1:2, 2:3 and 1:1 weight ratio). The formulation codes were designated as PV (~VETDSPV6, without coating), CH1PV (1:10 weight ratio), CH2PV (1:6 weight ratio), CH3PV (1:2 weight ratio), CH4PV (2:3 weight ratio) and CH5PV (1:1 weight ratio) for CH Pu coated liposomes and NV (~VETNV8), CH1NV, CH2NV, CH3NV, CH4NV and CH5NV for CH Pu coated niosomes. The formulation codes were SCH1PV, SCH2PV, SCH3PV, SCH4PV and SCH5PV for SCH Pu coated liposomes and SCH1NV, SCH2NV, SCH3NV, SCH4NV and SCH5NV for SCH Pu niosomes.

The surface charge of the vesicles was measured to ensure the completion of the coating. Simultaneously, zeta potential measurements were used to optimize the hydrophobized polysaccharide to lipid/surfactant ratio. Zeta potential values of vesicles were consistently stabilized at a ratio of CH Pu to lipid/surfactant (1:2 and 2:3) when measured the current with an applied voltage of 10 mV. On this basis formulations, CH3PV, CH4PV, SCH3PV (for liposomes) and CH3NV, CH4NV, SCH3NV (for niosomes) were selected for further evaluation. This could be due to the fact that CH Pu may fill the locus of defects by becoming a part of the vesicle membrane thus establishing equilibrium at this ratio of coat material. Optimized time for the coating of the vesicle bilayer was found to be 6 h, as no significant change was recorded in the zeta potential of coated systems beyond this time period. Once various process parameters were optimized, these were used for the coating with SCH Pu (sialo-pullulan).

Vesicles coated with hydrophobized pullulan were subjected to vesicle type, shape and size analysis with the help of electron microscopic techniques. Vesicles were found to be spherical and unilamellar with a slight plurilamellar fraction having mean diameters in the range of 400-450 nm. The mean vesicle sizes of liposomal formulations, i.e., PV, CH3PV, CH4PV, SCH3PV were found to be 412.7±31 nm, 437.6±42 nm, 454.2±81 nm and 467.1±52 nm, respectively. The mean vesicle sizes of niosomal formulations, i.e., NV, CH3NV, CH4NV, SCH3NV were found to be 412±31 nm, 447±43 nm, 456±87 nm, 483±55 nm, respectively. The coating was distinctively visualized as a continuous opaque surface over vesicles. Moreover, coated vesicles were relatively larger in mean size, as compared to plain vesicles (uncoated). This may be accounted to the polymeric capping, a secondary barrier structured on the vesicle surface.
% Encapsulation of metronidazole in pullulan coated liposomes, i.e., PV, CH3PV, CH4PV, SCH3PV was found to be 36.6±7.8, 32.9±9.8, 32.7±5.5 and 31.9±6.1 respectively. %EE of drug in pullulan coated niosomes, i.e., NV, CH3NV, CH4NV, SCH3NV was found to be 37.6 ±4.1, 32.1±6.7, 31.7±5.5 and 30.4±4.7 respectively.

This reveals that polysaccharide coating resulted into relatively lower levels of % EE as compared against 36.6±7.8 recorded for plain liposomes and 37.6±4.1 for plain niosomes. This could be attributed to the time course of coating required that might have allowed some fractions of drug leaching from the vesicles. The decrease in %EE was recorded statistically significant when compared with other (P< 0.05) in a rank sum test manner. To gain more information about the interaction of the delivery systems with harsh environments of biofluids and resultant stability, polysaccharide coated vesicles were subjected to various stability studies.

Release studies were performed using a dialysis bag and under sink (stirring) condition in PBS (pH 7.4). The relative release of drug was significantly suppressed on coating of vesicles with the polysaccharides. Plain vesicles released 10.6±3.5 % (for liposomes) and 9.4±1.5% (for niosomes) of drug in 180 min. The metronidazole release was nearly linear up to 6 h and subsequently a plateau was recorded. The cumulative drug release recorded for plain liposomes and niosomes after 12 h of release studies was recorded to be 22.3±5.6% and 21.4±6.5 % respectively.

Vesicles coated with hydrophobized polysaccharide were however, comparatively poorly permeable for solute and more than 25% lower cumulative drug release was recorded in first 180 min. CHPu and SCHPu coated liposomes released 6.8±1.6% and 5.1±0.9% %, whereas in case of polysaccharide coated niosomes, the cumulative drug release was 4.3±0.6% and 6.4±1.8% % respectively. However, more than 50 % decrease in cumulative drug release was estimated with for coated vesicles after 12 h as 10.25±4.1% (for CHPu coated) and 7.25±2.7% (for SCHPu coated) cumulative drug release was recorded for coated liposomes in the same time period as compared to 22.3±5.6% cumulative drug release recorded for plain liposomes. Similarly, 7.3±2.6% (for CHPu coated) and 9.7±0.5%% (for
SCHPu coated) cumulative drug release was recorded for coated niosomes as compared to 21.4±6.5 % recorded for plain niosomes during the same time period.

This clearly indicates that though coating with polysaccharides influences the release pattern but the nature of polysaccharides (CHPu versus SCHPu) do not make any significant effect on overall results. The difference in cumulative drug release between plain and coated versions was statistically significant (P<0.005) and could be due to the double barrier effect offered by vesicle membrane and polysaccharide coating.

8.2.2. Characterization of lectinized liposomes and niosomes

The tremendous capability of lectins (proteins) as ligands resides in the fact that they could impart an ability to resist the gut proteolysis and a high specificity towards carbohydrate determinant of the target sites.

Selected VET formulations, i.e. liposomes (DSPV6) and niosomes (NV8) were coated with Concanavalin-A (Con-A) using carbodiimide activated carboxylate groups of Con-A and underivatized PE of preformed vesicles. Underivatized PE in vesicle membrane contains an amine group that can participate in the carbodiimide reaction with carboxylate groups on lectin (Dunnick et al., 1975). The water-soluble carbodiimide EDC activates carboxylate groups on Con-A to form active-ester intermediates that reacts with PE to form an amide linkage. Elution profile of the system in sugar glyco-link column (Bio-Sorb™) reveals effective coating of metronidazole bearing PE-vesicle with Con-A. The coincidence of the peaks of lipid (Stewart assay, 1959) and protein (Lowry and Smith modified assay, 1975) at the void volume of the column and its distinctive separation from the peaks of unconjugated protein demonstrate the integrity of the proteoliposomes with encapsulated metronidazole. Similar elution profile was recorded for proteoniosomes with encapsulated metronidazole.

The degree of Con-A conjugation to PE-vesicles was assessed as a function of the carbodiimide concentration (amount of carbodiimide reagent added to the vesicle dispersion) and the reaction time (incubation time between the carbodiimide reagent and vesicle dispersion). The influence of the carbodiimide activation time on lectin attachment to the vesicle was assessed at a concentration of 1.0 mg carbodiimide reagent
(ethylenediamine carbodiimide, EDC) per ml of protein/lipid mixture. The results indicate an increase in the lectin conjugation with vesicles until a plateau level of 6 h, where equilibrium was established. At this optimized reaction time, a Con-A fixation efficiency of about 27.5% of the initial ligand bulk concentration was recorded. Similarly, ligand binding increased with the carbodiimide concentration. In this case a plateau could be reached at a reagent content of about 5.0 mg carbodiimide per ml of protein/lipid mixture. Based on these studies, the amount of bound lectin was calculated to be about 52.5 µg Con-A per ml of protein/lipid mixture. However, in the experiments carried out in the absence of carbodiimide, a small but detectable amount of lectin remained bound (2.51 µg lectin per ml of protein/lipid mix). This could be ascribed to the adsorption of lectin to the vesicle surface due to the hydrophobic or electrostatic interactions. The optimization was carried out in order to assure that the amount of EDC used in the coupling process should perform the anchoring of proteins to vesicles and should not be in excess so as to cause protein-protein cross-linking or protein precipitation. At an amount of 5.0 g per ml of protein/lipid mix, EDC did not cause any instability. Beyond this amount however, the vesicle aggregation and protein precipitation were evident. A parallel zeta potential experiment was also run in order to optimize the coating ratio at the optimized EDC concentration and reaction time values. For various weight ratio of Con-A with total lipid (1:10, 1:6, 1:2, 2:3 and 1:1 weight ratio) formulations were designated as C1PV, C2PV, C3PV, C4PV and C5PV (for liposomes) and C1NV, C2NV, C3NV, C4NV and C5NV (for niosomes) respectively. Surface charge stabilization as evident from zeta potential measurements revealed that a lectin to lipid weight ratio of 1:10 and 1:6, could stabilize the vesicles and based upon the results formulations C1PV and C2PV (for liposomes) and C1NV and C2NV (for niosomes) were selected for further studies.

Plain and protein coated vesicles were characterized using transmission electron microscopy and found to have an average vesicle size of 400 to 450 nm. However, no significant change was observed on coating of the vesicles with Con-A. The mean vesicle sizes of liposomal preparations, i.e., PV, C1PV and C2PV were found to be 412.7±31 nm, 414.5±11 nm and 421.5±67 nm respectively.
The mean vesicle sizes of niosomal preparations, i.e., NV, C1NV and C2NV were found to be 412±31 nm, 424.5±23 nm and 426.3±24 nm respectively. However, the lectinized vesicles appeared opaque, which may be accounted to protein coat. The % encapsulation of metronidazole in liposomes and niosomes was followed by separation of the unentrapped drug by centrifugation, and trapped drug in the pellets was estimated using UV at 320 nm following vesicle disruption using n-propanol: water (2:1 v/v).

% Encapsulation of lectinized liposomes, i.e., PV, C1PV and C2PV was found to be 36.6±7.8, 34.8±5.3 and 33.5±7.4 respectively. Lectinized niosomes, i.e., NV1, C1NV1 and C2NV1 recorded %EE of 37.6±4.1, 35.1±5.7 and 34.5±6.2 respectively.

% Encapsulation was marginally decreased in the coated vesicles compared to plain uncoated vesicles. The decrease in % encapsulation could be attributed to the residual drug leakage from the vesicles during the incubation time. Surface charge of the vesicles measured prior to and after the coating of the vesicle suggests for covalent coating as evident by quenching.

Lectinized vesicles were subjected for release studies in PBS (pH 7.4) under sink (stirring) condition. It was recorded that % cumulative drug released from lectinized liposomes and niosomes during the release experiments as measured in dialyzed fluids was low as compared against metronidazole loaded plain liposomes and niosomes (P<0.005). The % cumulative release after 12 h for plain liposomes and niosomes was 22.3±5.6% and 21.4±6.5 respectively, whereas after coating with Con-A, the % cumulative release was decreased and recorded to be 11.25±2.3 and 10.5±2.1% respectively. As in the case of polysaccharide coating, the dual diffusion barrier could be attributed to retarded release trend of encapsulated drug. Since the system is based upon ligand mediated localization of the entrapped drug, the retardant drug efflux should not influence its targeting potential.
8.2.3. *In vitro* ligand activity and specificity studies

In order to assess the suitability of the developed systems for biofilm targeting, the functionality of the anchored ligand in terms of their activity and affinity were evaluated.

8.2.3.1. *Polysaccharide coated liposomes and niosomes*

The coating and ligand activity of polysaccharides on the surface of liposomes and niosomes was confirmed qualitatively by the agglutination with soluble lectins. CHPu coated vesicles was agglutinated and peptized by soluble Concanavalin-A (Con-A) and SCHPu coated vesicles by wheat germ agglutinin (WGA). No significant aggregation was observed in vesicles without any polysaccharide coat (control). The extent of aggregation of the polysaccharide coated vesicles increased first with increasing amounts of hydrophobized pullulan used for coating. It was observed that in case of coated vesicles, the turbidity increased and then gradually leveled off. Lectin induced specific aggregation of the coated vesicles was completely coincided with the observations previously reported (Iwamoto et al., 1991; Sihorkar and Vyas, 1999). The results suggest that surface ligand anchoring and the process used did not affect the ligand affinity and selectivity for their recognition motifs. Con-A is one of the well-investigated lectins and is known to specifically bind mannose, fructose or glucose residues of polysaccharides or oligosaccharides complex structures. Similarly, WGA selectively bind N-acetyl neuraminic acid (sialic acid)-terminated oligosaccharide structures. These results are in accordance with the fact that the polysaccharides on the surface of vesicles behave as a cell specific ligand. Moreover, they are able to maintain their ligand activity against lectins even after being immobilized on the surface of vesicles. Con-A and WGA induced aggregation reveals the inter-digitization of cholesteroyl pullulan in to the vesicle bilayer whereby polysaccharide segment is projected towards the aqueous phase and expresses functional properties. Similarly, sialic acid segment anchored to the cholesteroyl pullulan (sialo-pullulan) was found to retain the specificity as demonstrated by WGA-induced aggregation of SCHPu coated vesicles.
8.2.3.2. Lectinized liposomes and niosomes

The ligand related activity was evaluated and established by bovine-maxillary-gland mucin (BSM) interaction with lectinized vesicles. Lectinized vesicles were put into contact with BSM, which was chosen as a biological model to determine the in vitro activity and specificity of the vesicle conjugated Con-A towards the sugar residues of the glycoprotein. BSM is a glycoprotein whose carbohydrate part is formed by oligosaccharide chains composed of six different sugars: N-acetyl galactosamine (69.2 μg/mg), N-acetyl glucosamine (168 μg/mg), galactose (15.2 μg/mg), mannose (2.07 μg/mg), fucose (9.53 μg/mg) and sialic acid (16.9 μg/mg). Experiments were carried out to evaluate the degree of functionality of vesicle conjugated lectin recorded after the addition of BSM, which expresses sugars for interaction. Experiments were performed in the absence of specific sugar for Con-A (for the study of in vitro activity) and in the presence of specific sugar, mannose (for the study of in vitro specificity).

Analogous to reported study, which describes that chemical modification of Con-A does not lead to any significant changes in lectin affinity towards saccharides (Bogdanov et al., 1989), no change in the ligand activity and affinity of Con-A (immobilized on vesicle bilayer) was detected. Plain liposomes recorded 1.71%, 1.69, 1.89 and 1.77, where as plain niosomes recorded 1.94%, 1.87%, 2.13% and 2.01% BSM binding. The results remained statistically insignificant either in the presence or absence of the competing sugar (α-methyl mannoside) for Con-A. Protein coated liposomes, however recorded % BSM binding of 4.86 in the absence and 1.21% in the presence of α-methyl mannoside. The results were more pronounced when 10 mM Ca ++ was added to the reaction mixture and respective values 22.1% and 7.9% respectively were recorded in the absence and presence of α-methyl mannoside. Similarly, lectinized niosomes recorded 24.6% BSM binding in the absence and 4.5% in the presence of α-methyl mannoside. From these results, it could be ascertained that in the absence of α-methyl mannoside, lectinized systems showed more than two-fold level of interaction with BSM as compared to the interaction recorded for uncoated (plain) vesicles (used as control). These results clearly indicate that vesicle conjugated lectin remained functionally active.
In the case of plain liposomes (control) the results remained unvaried for vesicle-mucin interaction irrespective of the presence or absence of the specific sugar. However, the marginal % BSM interaction exhibited by plain vesicles could be ascribed to non-specific adsorption of protein (BSM) over vesicles surface. In contrast, the degree of interaction between lectinized system and mucin decreased significantly on addition of competitive sugar. The results clearly reveal that lectinized vesicles possess the sugar specificity that corresponds to the native lectin.

The addition of 10 mM Ca\(^{++}\) to the reaction mixture is a prerequisite for the Con-A sugar recognition, which is a well established characteristics (Farajollahi et al., 1998). Ligand anchored liposomal and niosomal VETs were further subjected to various pharmaceutical and biological stability studies.

**8.3. STABILITY OF LIPOSOMAL AND NIOSOMAL SYSTEMS**

Liposomes and niosomes were investigated intensively for oral or peroral delivery of drugs. The present study was also aimed at making an improved and stable (as compared against conventional vesicles) and osmotically active (in case of niosomes) vehicle especially for oral delivery of water-soluble anti-bacterial drug. However, in order to be effective as carriers for the oral administration of bioactives, they must be able to withstand bioenvironmental challenges encountered during passage through the oral cavity, stomach and small intestine.

In the present study, plain and coated liposomes were studied for stability under varied pH conditions, against bile salts and pancreatic lipases and on long-term storage. Plain and coated niosomes were subjected to osmotic stress, detergent challenge, freeze thawing and long term storage studies.

**8.3.1. Influence of polysaccharide coating on stability of liposomes and niosomes**

Polysaccharide coated liposomes were tested *in vitro* for their stability in the presence of bile salts, pancreatic lipase, and variations in pH. While low pH (~1.2-2.5) caused aggregation in the case of plain liposomes, it had rather less effect on coated liposomes.
Similarly, addition of more than 9 mM bile salts facilitated the release of drugs as it was exceeded to over 80% from plain liposomes except those coated with hydrophobized polysaccharide. However, the polysaccharide-coated vesicles were resistant to pancreatic lipase alone or in combination with bile salts (synthetic intestine) and therefore may be suitable for use in the oral administration of therapeutic agents.

The influence of pH variations from 1.5-2.5 (stomach) to 7.4 (intestine) at physiological temperature (37° C) was investigated. Acidification of plain liposome dispersion induced instantaneous vesicle aggregation, which was partially reversible when the external medium was neutralized. Simultaneously, complex morphological bilayer rearrangements occurred, leading to the formation of small aggregates. Polysaccharide coated vesicles however exhibited exceptional stability towards acidic pH. % Residual drug content recorded for plain liposomes was 23.12±3.4% at pH 2.5, whereas coated versions retained more than 75% of the drug (84.23±6.9% for CHPu coated and 83.19±9.2% for SCHPU coated liposomes) during 12 h incubation period with Hydrochloric Acid Buffer (HAB, pH 2.5). Though as the pH of the exposure solution was increased from 1.2 to 7.4, the differences in % residual content values were less prominent amongst plain and coated formulations. However, coated liposomes recorded higher values of % residual drug content at all pH solutions tested.

Similarly, experiments were carried out to check stability of osmotically active niosomal formulations. The osmotic activity of niosomes was checked after being coated with CHPu and SCHPU. The vesicles integrity of hydrophobized polysaccharide coated niosomes was assessed after subjecting them to an osmotic stress under increasing concentration of different molar solutions of sodium chloride. Polysaccharide coated vesicles were found to resist the tonicity gradiance. The average vesicle size was increased under hypotonic environment, whilst under hypertonic conditions a marginal shrinkage and resultant decrease in the average size was observed. Polysaccharide coated niosomes though exhibited resistance towards different osmotic strength solution of sodium chloride, they recorded an increased size in hypotonic environment, however the increase in size was found to be marginal. It may be due to the hydrophilic and polymeric nature of polysaccharide ligands, which may imbibe water and swell thus increasing the
size of the vesicles. CHPu and SCHPu coated niosomes exhibited insignificant changes in vesicle morphology. The findings reflect better vesicle integrity against the osmotic challenges.

Another apparent effect of webbing hydrophobized pullulan on to the bilayer membrane was the protection against bile salt (liposomes) and detergent (niosomes) solubilization of the system and encapsulated drug.

Stability of liposomal formulations were tested by incubating vesicles with different molar concentration of bile salts/detergent (below, above and in the vicinity of Critical Micelle Concentration) and with fresh rat bile at 37±1°C for a period of 6 hours. In all stability experiments with different bile salts at various concentrations, no significant changes in the vesicle size, integrity and drug content were observed over 3 h incubation periods for coated liposomes. The same holds for the rat bile incubation studies. The effects of bile salts and lipases on the liposome structures were also studied. It was recorded that plain liposomes lost all their contents and disrupted at or beyond 12 mM taurocholate solution, whereas polysaccharide coated liposomes resisted membrane disruption even on exposure upto 15 mM of taurocholate solution. Membrane solubilization by bile salts was favored by preliminary liposome incubation in acid conditions. However in the case of coated liposomes, in spite of slight morphological modifications, vesicle structures were retained even after an acid stress. Pancreatic lipase and simulated human bile had synergistic effect on the release of drug from plain vesicles but not from the polysaccharide-coated vesicles.

In a parallel experiment for niosomes, the relative stability of the coated vesicles was measured in terms of % residual drug content following challenges of detergent, decyl-PEG. Free (unentrapped) drug was removed by chromatography of the vesicles on Sephadex G-50 column prior to the experiment. Addition of the detergent decyl-PEG300 caused a rapid release of entrapped drug on its addition beyond 9 mM indicating the structure defects in the bilayer membrane. These results are in accordance with the membrane solubilization reported by Ucheghu and Vyas, 1998. The solubilization of bilayer via the build up of CMC of detergent molecules within the niosomal membrane,
followed by micellization could be proposed as the possible mechanism for the vesicle disruption at higher bile salt concentrations. Coating of the vesicles with hydrophobized pullulan however severely retarded the rate of detergent induced release. Niosomes after coating with polysaccharides demonstrated better stability as compared to plain niosomal preparations. It could be seen that at detergent concentrations below and at CMC, the drug content was not affected for coated versions. However, in the case of plain niosomes, at higher concentrations of detergent above 9 mM, solubilization of bilayer could be proposed for the leaching of the drug. Dual diffusion barrier of coated niosomes could be proposed as a possible explanation for their stability against the detergent challenges.

Freezing of vesicles in liquid nitrogen, and subsequent thawing at room temperature, normally leads to a collapse of the vesicles and induce fusion of the membranes due to dehydration. Consequently, freeze thawing of vesicle results in nearly complete release of the encapsulated contents. Plain niosomes lost their contents after the stress challenge (100% value). At the optimum ligand concentration, 81-84±2.4% liposomal population and 90-92±1.7% niosomal population were observed to be intact, whereas the same concentration of underivatized pullulan (associated to vesicle membrane by adsorption) could not offer an appreciable protection to either plain liposomes or niosomes.

Long term stability is critical for pharmaceutical significance of finished formulations. Long term stability of vesicles was examined by measuring the spontaneous leaching of encapsulated contents on the storage in vessels without stirring and as a function of time at an ambient temperature. Polysaccharide coated liposomes recorded 7.5±2.5 leaching at the end of first month as compared against nearly 20% leaching from plain liposomes. Subsequently, the loss of drug from coated versions was negligible indicating the ability of the system to withstand across membrane osmotic gradiance thus retains better packing of membrane resulting in to effective trapping efficiency of the system. The nature of hydrophobized anchor (CHPu versus SCHPu) didn’t reflect any appreciable change in the stability profile.
Plain niosomes were also stable as compared against plain liposomes and recorded only 11.1±1.6 % leakage in first month. It was seen that a large population of liposomes lost their integrity and 70.8±4.2 % of drug leakage occurred after 6 months of storage at an ambient temperature. At the end of the long-term stability experiments, the majority of plain liposomes found to be disrupted. On the other hand, plain niosomes recorded nearly 40% leaching of the drug, indicating their potential as putative oral drug delivery systems. Coated versions, however recorded exceptional shelf stability (in 6 months) as CHPu coated liposomes (76.5±3.5%), CHPu coated niosomes (85.1±3.7%), SCHPu coated liposomes (81.1±2.7%) and SCHPu coated niosomes (86.7±3.8%) recorded better results as compared against plain liposomes (30.1±2.4%) and plain niosomes (61.5±3.9%). Similar trend was observed at a lower, i.e., 4±1°C storage. However, an exceptionally low leaching profile was recorded, as appreciated due to higher stability of the vesicular membranes at lower temperatures.

8.3.2. Influence of lectinization on the stability of liposomes and niosomes

Similar experiments were performed with Con-A coated liposomes and niosomes as performed for the polysaccharide coated vesicles, i.e., exposure to different pH environments, bile salts and lipases and long term storage (for liposomes) and osmotic stress, detergent action and long-term storage (for niosomes).

Effects of various pH environments (ranging from pH 1.2 to pH 7.4) on the residual drug content of Con-A coated liposomes and plain liposomes were studied and compared. Whereas polysaccharide-coated liposomes recorded nearly 3 times higher % residual drug content (~75%), Con-A coated liposomes recorded nearly 2.5 times (~67.5%) higher values as compared against plain liposomes (~25%).

In order to assess the stability of the lectinized system against various osmotic environments as encountered in the biological milieu after oral delivery, the changes in vesicle size after being exposed to various osmotic stress conditions were monitored. Plain niosomes when incubated with different osmotic environments recorded average vesicles diameter of 355 (control), 605 (50 mM of NaCl), 345 (100 mM of NaCl), 192
(150 mM of NaCl), 134 (200 mM of NaCl) and 120 nm (250 mM of NaCl) respectively. Whereas lectinized niosomes recorded 412, 446, 436, 411, 409 and 391 nm after incubation in 0 (control), 50, 100, 150, 200 and 250 mM of NaCl solution receptively. The results clearly demonstrate the importance of protein coating (a dual barrier) on niosomal permeability characteristics. The results show that protein free niosomes are lesser stable compared to lectinized niosomes. Whereas plain niosomes were highly osmotically active, the coated versions (Con-A coated) demonstrated no change in vesicle size on exposing the dispersion to hyper or hypotonic solutions. Unlike polysaccharide coated niosomes, no significant size variation was recorded for Con-A coated niosomes under hypotonic environment.

Similar to results obtained with polysaccharide-coated vesicles, lectinized vesicles offered exceptional resistance to bile salts (against liposomes) and detergent (against niosomes) action. Long-term stability experiments indicated an improved resistance to the fusion or aggregation normally encountered in plain vesicles. Lectinized liposomes showed a residual drug content of 75.57±4.5% and lectinized niosomes of 87.14±3.7% at the end of six-month incubation period, whereas plain vesicles showed 30.1±2.4% (for liposomes) and 61.5±3.9% (for niosomes) respectively. These results indicate that the coating of vesicles with lectins offers them stability.

The hydrophobized pullulan and Con-A coated (lectinized) vesicles thus exhibit exceptional stability profile against the challenge of pH, ionic strength, osmotic pressure, simulated enzymatic solutions and long term stability along with suppressed leakage of entrapped drugs. With an advantage of superior stability over liposomes, niosomes definitely have an edge, which could further be strengthened by coating with these ligands. It could be inferred that the anchoring of ligands make these vesicles suitable for oral/peroral delivery of biocides or antimicrobial agents.

8.4. BIOFILM TARGETING OFFERED BY ENGINEERED VESICULAR CONSTRUCTS

The most important part of the present study was to ascertain the effectiveness of the developed systems for biofilm targeting. To achieve this goal, various *in vitro*, *ex vivo*
and in vivo biofilm models were developed and targeting potential of ligand anchored vesicular systems was investigated. Staphylococcus aureus, is the causative organism for the skin-associated, implant-associated and gastrointestinal mucosa-associated biofilm infection (gastroenteritis and food poisoning) and intoxication. In the present study, we established through in vivo model that S. aureus forms biofilm during gastrointestinal infections on damaged mucosa. With the failure of conventional means to achieve therapeutic levels after oral administration for gastrointestinal biofilm infections, the study performed was aimed at further elaboration of targeting potential of the developed systems against various in vitro, ex vivo and in vivo biofilm models.

Two biofilm models of S. aureus were developed in vitro, one on cellulose filter paper (to check formation of S. aureus biofilms by SEM) and the other on microtitre plate (to investigate biofilm targeting efficiency). Biofilms were deposited and fixed with glutaraldehyde cross-linking on cellulose filter paper (filter paper biofilm model). Microtitre plate (Sihorkar and Vyas, 2001) biofilm model of S. aureus were developed and used in our study as an in vitro model to assess the efficacy of the formulations using a regrowth assay.

Ex vivo biofilm model of S. aureus was developed using a rat pouch infected site and formulations were assessed in terms of their ability to offer reduction in the % bacterial viability.

In vivo biofilm model was developed in the intestine of a rat infected with S. aureus and the localization of developed formulation was assessed using fluorescent microscopy of the wax embedded frozen intestine sections.

8.4.1. In vitro model for the assessment of biofilm targeting

Vesicular systems (formulations, CH3PV, SCH3PV, CH3NV, SCH3NV, C1NV and C1PV were selected for biofilm targeting assay) were investigated for their biofilm targeting potential. Scanning electron microscopy further confirms the formation of biofilm of S. aureus on the surface of membrane filters. Dense colonization of bacteria and thick membranous or fibrous structures that cover the colonies were observed.
In a parallel experiment, the biofilm targeting potential of developed formulations was assessed in terms of % biofilm growth inhibition in a regrowth assay against *S. aureus* developed on an *in vitro* developed microtitre plate model. % Biofilm growth inhibition (% BGI), i.e., the ratio of increase in absorbance at 630 nm (A630) of a formulation against that of wells containing *S. aureus* alone, resulted due to different formulations was measured, which served as an index of biofilm targeting.

Various vesicular systems containing metronidazole in an equivalent concentration corresponding to MIC values of 50%, 75% and 90% against *S. aureus* were subjected to % BGI studies. Parallel experiments were carried out, where drug free vesicular systems were used to assess whether carrier systems and ligands themselves contribute to the biofilm growth inhibition. It was noted that free drug exhibited % BGI values in a dose-dependent manner. The same holds for plain liposomes and niosomes, where drug diffuses out under a concentration gradiance and the loaded drug thus becomes available only after getting released. Coated liposomes and niosomes however remain nearly unaffected to a dose level beyond 50% MIC as the systems interacted with cellular carbohydrate/lectin and thus *en route* to cellular interior and thus exerting dose-independent activities.

The efficiency of targeting of lectinized liposomes to *S. aureus* biofilms has been measured in terms of % BGI. The values recorded for plain drug, plain liposomes, lectinized liposomes, CHPu coated liposomes and SCHPu coated liposomes at 75% MIC (corresponding metronidazole concentration) level were found to be 55.1±4%, 64±4%, 96.5±2%, 90.1±3% and 75.7±5% respectively. Similarly, the values recorded for various niosomes were 71.6±4% (plain niosomes), 95.3±2% (lectinized niosomes), 69.6±6% (CHPu coated niosomes) and 87.3±4% (SCHPu coated niosomes). However, the values recorded for plain liposomal and niosomal systems were higher for long-term incubation periods (~12 h) but they were still lower than those recorded for ligand anchored formulation even after shorter exposure or incubation periods (2 h).

The effect shown by plain vesicles (both liposomes and niosomes) on long term incubation (6 h as compared against 2 h of coated liposomes) could be ascribed to the
slow leakage of the entrapped content. This suggests that these vesicles affected the bacteria by releasing their contents via leakage through the bilayer membrane into the surrounding medium, where the drug could come in contact with the biofilm. However, % encapsulation of metronidazole within plain liposomes and niosomes lowered the antimicrobial activity of metronidazole because the effective level of the agent could not be attained when drug was made available from entrapped pool. This is due to the fact that a major proportion of the drug would have remained inside the aqueous core of the vesicles, whereas a fraction of free drug to which the biofilm exposed was insufficient (to be above MIC).

Lectinized liposomes and niosomes were superior compared to plain liposomes and free drug in exhibiting a higher value of % BGI over a period of 1 h. It was gradually leveled off signifying the higher and sustained % growth inhibition values for coated module. The superiority of lectin coated vesicles indicates that attachment of the vesicles to the bacterial bio-film by anchored exopolysaccharide polymer segment of the Con-A could prove to be a key factor in exhibiting higher % BGI values.

The enhancement of antimicrobial activity when using lectin-coated liposomes and niosomes was most probably due to their ability to adsorb/adhere or fuse with the bacterial bio-film due to ligand mediated interactions and subsequently releasing all or a part of antimicrobial agent within or in the vicinity of the target. These ligand-mediated interactions provide exceptional advantages over the free drug and metronidazole bearing plain vesicles. Firstly, because the lectin-coated vesicles were in direct proximity with the bio-film surface, the released drug would have a better probability for diffusion into the bio-film than the free circulating drug. Secondly, unlike the free drug, the adsorbed vesicles were retained at the biofilm after the incubation period and released their contents into the vicinity of biofilm surface during the 24 h regrowth assay.

A ligand target enhancement (LTE) factor was calculated for a range of polysaccharide coated (polysaccharide target enhancement) and lectin coated (lectin target enhancement) vesicles, subjected to targeting assay against biofilms of Staphylococcus aureus. LTE denotes the extent of growth inhibition of biofilm on a
microtitre plate model by ligand coated liposomes relative to that of ligand-free liposomes. This was chosen as an indicator of the degree of increase in targetability contributed by the ligands anchored on the vesicle surface. In all the investigated systems, the lectinized vesicles (both liposomes and niosomes) targeted effectively relative to lectin free liposomes of the same composition. Lectinized liposomes (C1PV and C2PV) recorded LTE values of 6.01 and 5.96, whereas lectinized niosomes (C1NV and C2NV) recorded LTE values of 4.02 and 4.06 respectively. Polysaccharide coated liposomes on the other hand, provided appreciable improvement as compared against their plain counterparts, however they were not superior to lectinized systems. The values of LTE contributed by pullulan coated liposomes (CH3PV and CH4PV) were 2.39 and 2.34 respectively. The same trend was followed with ligand-anchored niosomes and their plain version. The values of LTE for pullulan coated niosomes (CH3NV and CH4NV) were recorded to be 1.96 and 2.01 respectively. However, an increase in the targeting efficiency was recorded when sialic acid was conjugated to pullulan (sialo-pullulan) and anchored as ligand on vesicles. Sialo-pullulan (SCHPu) coated liposomes recorded an enhanced LTE value of 4.24. The values of LTE for SCHPu coated niosomes (SCH3NV) was recorded to be 3.68.

8.4.2. Ex vivo model for the assessment of biofilm targeting

The efficacy of the developed vesicular systems was assessed in an ex vivo developed biofilm model, in order to quantify the potential of these systems in lowering down the bacterial load of the infection. Ex vivo targeting using ligand-anchored liposomes and niosomes was demonstrated in terms of % remaining viable counts of S. aureus biofilm developed in rat pouch (Ajiki et al., 1991).

The model was fabricated by injecting 10 ml of air subcutaneously on the back of the rat with a 21-gauge needle followed by aspiration of the air in the pouch. Subsequently, 10 ml of sterilized CMC (1.5% in saline) was injected and an infection was induced by inoculating $10^6$ CFU of Staphylococcus aureus per pouch along with the injection of CMC. The therapy with drug (control), plain liposomes/niosomes and their
coated version began 4 days after the incubation, with oral administration of the dispersions (equivalent to 100 mg per Kg per dose). The numbers of viable counts of bacteria in the pouch exudates were counted using established procedures (Ajiki et al., 1991).

Plain liposomes, plain niosomes, lectinized versions of liposomes and niosomes and polysaccharide-coated liposomes and niosomes were compared for their efficacy against _S. aureus_ biofilms developed _in vivo_ in the rat pouch. The effect of plain drug in the planktonic phase (suspension culture) and biofilm phase was also compared.

The effects of antimicrobials agent towards bacteria harboring as a biofilm as against fluid phase (plankton) population were exceptionally different. Whereas the plain drug could bring a 1 log\(_{10}\) (CFU/ml) reduction in the % bacterial viability in biofilm phase, a 3 log\(_{10}\) reduction in % viability could be recorded with the suspension phase. Moreover, as in the case of plain drug administered to the bacteria in the fluid phase (plankton, suspension culture), the increase in the total viable count after the first day was significantly low compared to plain drug when administered to bacterial biofilm (in pouch biofilm infection model). This may account for the drug resistance encountered in the treatment of oral or intestinal bacteria, which on the laboratory scale and in fluid phase always provide good results.

Metronidazole loaded liposomal and niosomal formulations were tested in a pouch infection model for a period of six days. The formulations were administered every day and the pouch exudates were tested for the % reduction in the viability of bacteria. A 4 log\(_{10}\) reduction was recorded with lectinized systems (liposomes and niosomes) as compared against 1 log\(_{10}\) reduction with their plain counterparts. However, the reverse pattern of increase in number of viable cells observed with the administration of plain niosomes and liposomes, could not be observed for lectinized system. This signifies that the release of the drug is ligand-mediated and not merely the diffusion controlled phenomenon thus causing the increased localized concentration in the vicinity and the interiors of the biofilm, leading to a higher and sustained antibacterial effect. The similar mechanisms as mentioned with the % BGI studies could be accounted here as well.
On the other hand, an interesting observation that polysaccharide coating could not bring the effects to the similar degree as observed with lectinized systems. There was no significant difference in the efficiency of plain and pullulan coated vesicles. However, a 2 log_{10} reduction was recorded when sialic acid conjugated pullulan was used as a ligand. This clearly reveals that the plain liposomes and niosomes though provide better sustained effects as compared against plain drug, are less effective than ligand-anchored liposomes and niosomes.

Polysaccharides (pullulan) alone, however provide a comparable effect as imparted by plain vesicles. Ligands like lectin and sialic acid, may recognize the counter ligands expressed on the biofilm 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.

8.4.3. In vivo model for the assessment of biofilm targeting

The aim of this study was to ascertain the uptake of developed systems by the infected cells of the intestine harboring biofilms on their mucosal and sub-mucosal regions. This is a prime area of concern as far as the biofilm colonization associated with the gastrointestinal infection and intoxication is concerned. This would indirectly provide evidence about the efficacy of the systems on in vivo administration. The in vivo model was designed with a view to ascertain the in vivo applicability of the otherwise well proved in vitro targeted systems.

An infected (intoxicated) rat intestine biofilm model was developed in vivo. Since biofilm infections harbor preferentially over infected sites, the model was infected before colonization of bacterial biofilms. Necrotizing agent, indomethacin (1.0 % w/v, 1.0 ml, per Kg) was orally administered to rats while water was administered ad libitum for 24 h before the administration of bacterial suspension. This was followed by oral administration of bacterial suspension of Staphylococcus aureus (1×10^6 CFU) thrice a day for 2 days. Various formulations containing 6-carboxyfluorescein (6-CF) were administered with the help of feeding canula and after a period of 6 h, rats were
sacrificed. The localization of developed formulations was assessed using fluorescent microscopy of the wax embedded frozen intestine transverse sections (7μ thickness). The sections were observed under fluorescent microscope at excitation wavelength of 470 nm and emission wavelength of 560 nm for the presence of fluorescence. Washings of the intestine of the infected rats after sacrificing were passed through the membrane filter and fixed with glutaraldehyde and subjected to scanning electron microscopy.

*Staphylococcus aureus* has been proved as the causative organism for the gastroenteritis and food poisoning. In our study, we developed an infected rat intestine model and established for the first time that enterotoxin producing *Staphylococcus aureus* exist as biofilm on intestinal mucous. Scanning electron microscopy photograph of intestine associated infections revealed the existence of biofilms with associated polysaccharide slime.

Fluorescence microscopy study revealed that the prepared systems were well taken up by the epithelial cells as well as the mucosal regions of the intestine. However, it was noted that the intensity of the fluorescence was high in case of lectinized liposomes and niosomes. This could be attributed to preferential role of lectin (Con-A) as complementary ligand for surface carbohydrates of *Staphylococcal* biofilms. However, it must be stated at this point that the nature and type of biofilms may influence the uptake of ligand anchored systems and this fact should be considered while devising the stable oral delivery systems for biofilm targeting.

The superiority of polysaccharide coated vesicles as compared to plain vesicles could be ascribed to the interaction between the polyhydroxy –OH terminal groups available in the polysaccharides and the surface polymers (probably via hydrogen bonding with monosaccharides) of the bacterial glyco-calyx (Jones et al., 1994). Further, polysaccharide anchored sialic acid (which serve as ligand for the lectin receptors expressed on bacterial biofilm) was studied as an alternative to plain polysaccharide as a ligand. The fact that sialo-polysaccharide coated vesicles were superior in exhibiting better results indicates that attachment of the vesicles to the sialic acid specific lectins expressed on bacterial bio-film could be a key factor.
The multi- or polyvalency characteristics of the polysaccharides and lectin, i.e., binding to a target site through multiple interactions, originally proposed by Matrosovich (1989) could be used to partially explain the enhanced activity of coated module.

Lectin (immobilized on the surface of liposomes) may simply arrest glyco-calyx covering (glycoprotein and glycosphingolipids) through its multivalency and may cause peptization and sequestration of bio-films. Thus ligand-mediated bioevents could be accounted for the significant biofilm targeting potential offered by physicochemically and mechanically stable engineered vesicles.

8.5. CONCLUSIONS

Vesicular systems are versatile carriers for oral/peroral delivery of drugs and could prove beneficial for biofilm targeting of antibacterial drugs. However, in the unmodified form (without ligand anchoring) these vesicles merely protect the encapsulated drug to the extent where they withstand and remain intact against the stresses and challenges encountered in the mouth cavity and gastrointestinal tract. This however protects the drug from bioenvironmental challenges to an extent but once the system is destabilized, the entire loaded drug is exposed to the harsh environment. Similar is the problem of leaching of the water-soluble drugs or drugs with biphasic solubility from these vesicles. In order to obviate these drawbacks, we developed ligand-anchored vesicular systems. These ligands are not only aimed at providing the stability to the vesicles but also serve to present ligands to carbohydrate(s) expressed on the surface of the biofilms to negotiate drug targeting.

Following conclusions could be drawn from the concluded study:

1. Vesicles prepared using extrusion technique (VETs) were selected for the study due to their uniform size-distribution and homogeneity, with increased reproducibility of results.

2. Vesicles exhibited comparative morphological and physicochemical features prior to and after coating with polysaccharide and lectin ligands, i.e., cholesteryl pullulan (CHPu), sialic acid modified cholesteryl pullulan (SCHPu) and Concanavalin-A.
3. The original rate of drug release through plain liposomes and niosomes was significantly reduced to a slow and delayed release profile after coating of the vesicles.

4. Vesicles (both liposomes and niosomes) in the coated from proved exceptionally stable against the challenges of pH, bile salts and lipase, osmotic pressure, detergent, freeze thawing, detergent action and long term storage.

5. Biofilms associated with damaged and infected and intoxicated gastrointestinal mucosa (*Staphylococcus aureus* biofilms) as established in our study, could be effectively targeted using developed systems as ascertained through *in vitro* (micro titre plate model), *ex vivo* (rat pouch model) and *in vivo* (infected intestine biofilm model) models.

6. All developed systems exhibited exceptional *in vitro* targeting potential as compared against plain antibacterial drug solution against biofilm of *S. aureus* developed on micro titre plate model. However, lectinized liposomes and niosomes and sialo-pullulan coated vesicles provided the best results. Plain and pullulan coated vesicles demonstrated comparable results. Drug free vesicles (plain and coated) did not provide any antibacterial activity by themselves.

7. In *ex vivo* biofilm targeting studies, lectinized and sialo-pullulan coated vesicles provided the best results in terms of reduction in the % viability of the remaining bacterial load in the rat pouch after an initial infection with *S. aureus*.

8. In *in vivo* infected intestine rat model, significant localization of lectinized vesicles over the target site (of biofilm infections) was further confirmed using fluorescent microscopy.

In the light of these inferences, it can be concluded that the developed system(s) offer defined strategies in alleviating drug bacterial resistance problems, associated with bio-film colonization of the causative organism. They could also be used clinically for bacterial infections in the conditions like gastrointestinal and mucosal infections associated with gastritis, gastric ulcer and gastroenteritis. Furthermore, they may also find their application in the development of orally stable drug delivery system for periodontal pocket biofilm infections (dental plaque) and as topical delivery system for vaginitis and skin associated biofilm infections (catheter and implant associated biofilm infections).