Engineered vesicular constructs for biofilm targeting

BIOFILM TARGETING USING *IN VIVO* MODEL
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 infections is concerned. This would indirectly provide evidence about the efficacy of the systems on *in vivo* administration. The *in vivo* model was designed to ascertain and evaluate the performance of the developed system.

### 7.1. FORMATION OF INFECTED INTESTINE BIOFILM MODEL

An infected 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. Ulcerogenic agent, indomethacin (25 mg per Kg body weight) was orally administered to rats. This was followed by oral administration of bacterial suspension of *Staphylococci* (1×10^6 CFU) thrice a day for 2 days. Various formulations loaded with 6-carboxyfluorescein (6-CF) marker were administered and after a period of 6 h, rats were sacrificed. The localization of developed formulations was assessed using fluorescence microscopy of the wax embedded frozen intestine segments.

### 7.2. SCANNING ELECTRON MICROSCOPY OF THE BIOFILMS DEVELOPED AS INFECTED INTESTINE BIOFILM MODEL

Washings of the intestine of the infected rats after sacrificing them were passed through the membrane filter remains on the paper were fixed with glutaraldehyde and subjected to scanning electron microscopy. Similarly pieces of intestinal segments were also subjected to scanning electron microscopic studies. Samples (intestinal segments and membrane filters) were processed for scanning microscopy by fixation for 90 min at 4°C in 2% glutaraldehyde in 0.1 M saline phosphate buffer (pH 7.4) with sucrose 8%. This was followed by re-fixation at 4°C in 2% osmic acid in the same buffer, dehydration in a series of aqueous ethanol solutions (25%, 50%, 75% and 100%). These dehydrated filters were then dried using critical point dryer and then coated with platinum-palladium by using an ion coater and examined by using scanning electron microscope (Photomicrograph 15 and 16).
Photomicrograph 15: SEM of *S. aureus* biofilms deposited on infected intestine model (×1,000,000)

Photomicrograph 16: SEM of *S. aureus* biofilms deposited on infected intestine model after treatment with drug loaded vesicles (×17,500)
7.3. FLUORESCENCE MICROSCOPY OF THE BIOFILM INFECTED INTESTINE MODEL

Intestine was selected as the target organ as *Staphylococci* harbor in the intestine in the form of biofilms as ascertained in our study. However, it was realized that developed systems should support *in vivo*, the results recorded and hypothesis proposed during their *in vitro* and *ex vivo* biofilm-targeting studies.

Albino rats of Wister origin, weighing 150-200g were taken and divided in eight groups of 3 rats each. These rats were used to prepare infected intestine biofilm model as previously described. Formulations were given to albino rats with infected intestine biofilm model. Rats were sacrificed after 6 h, and intestine of these experimental rats was removed, cut along the curvature and the contents were washed with Ringer’s solution and dried using tissue paper. These pieces of intestine were removed from the Canary’s fluid (absolute alcohol:chloroform, 3:1v/v) for 3-4 h and subsequently dehydrated using absolute alcohol. After three changes of dehydrating solvent, intestine pieces were transferred in a mixture of absolute alcohol:xylene (1:1 v/v) for 15-30 min. After decanting the solvent, these intestinal pieces were then placed in xylene saturated with natural paraffin wax and kept overnight.

The next step was paraffin infiltration and embedding of the intestine segments. The matured wax was filtered to remove any suspended particles and was kept in the molten state for 24h at 60-65 °C. Intestine segments were transferred directly in molten wax in the first filtration unit for 30 min at 65°C in an oven. After the first embedding, intestine pieces were removed and placed in second infiltration and third infiltration, each for 30-min period. This was followed by block preparation of the intestine segments.

Blocks were prepared using Cuffling’s jar. Filtered matured wax was poured in the lid of Cuffling’s jar upto 4/5th of total height. The intestine segments were immediately removed from the infiltration unit and placed gently into the lid. It was allowed to stand at room temperature till solidification. The lid was placed in a tray containing fresh water. The block was cut and trimmed to remove excess wax. The prepared blocks were then cut in to ribbons of sections with the help of microtome.
Photomicrograph 17: Fluorescence image of intestine showing the localization achieved with lectinized liposomes

Photomicrograph 18: Fluorescence image of intestine showing the localization achieved with lectinized niosomes
Photomicrograph 19: Fluorescence image of intestine showing the uptake of lectinized liposomes in Peyers' patches

Photomicrograph 20: Fluorescence image of intestine showing uptake in Peyers' patches achieved with lectinized niosomes
Photomicrograph 21: Fluorescence image of intestine showing submucosal localization achieved with lectinized liposomes
transverse ribbon sections were transferred to a slide on which a fixative (0.01 ml of 1% w/v egg albumin solution) has been pre-applied and this slide was observed under a fluorescent microscope (Polymass, London). Fluorescence images of the biofilm targeted liposomes and niosomes are presented in Photomicrographs 17-21.

7.4. RESULTS AND DISCUSSION

Bacterial biofilms on the living tissues that result in chronic or refractory infections are probably constructed on mucosal or other bio-surfaces or biomaterials by way of complicating mechanisms. Many investigators reported *in vitro* experimental models of bacterial biofilms on the microtitre plates, silicone catheter tubing, cellulose acetate membrane and the surface of other devices by culturing bacteria in an artificial medium. Ideal properties sought in *in vitro* bacterial biofilm model are uniform film formation, uniform reproducibility and the ability to be investigated quantitatively. However, in *in vitro* model, an absolute mimic of highly complicated *in vivo* conditions is desirable. *In vitro* models though informative does not give real bio-architecture of biofilms, which is composed of bacteria, bacterial exopolymers, various insoluble proteins and glycoproteins and blood cells. In an attempt to overcome these drawbacks, an *in vivo* model was developed in the present study. Generally, it takes several days to form a biofilm in an infected tissue or organ, and it may take several days to investigate the therapeutic effect of drugs (and drug-loaded delivery systems) after the biofilm mode of growth of the infected bacteria is established.

Scanning electron microscopy studies revealed the formation of *Staphylococci* biofilms on infected rat intestine model. Abundant membranous structures and fibrous coat in the photomicrograph 15 indicates the formation of biofilms of *Staphylococcus aureus* in the infected intestine model. These viscous coverings that tie the bacterial colonies could be assumed as polymeric slime substances (exopolysaccharides), which are the major obstacle for the penetration of anti-bacterial agents through conventional delivery systems. Photomicrograph 16 records the SEM of biofilms after the treatment with drug loaded vesicles. It could be seen that the quantity of exopolysaccharides and
fibrous structure was reduced, which may lead to effective and therapeutic localization of the administered systems.

Fluorescence microscopy (Photomicrograph 17-21) study revealed that the prepared systems were well taken up by the mucosal as well as the sub-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 *Staphylococci* biofilms. Moreover, the system was also found to be localized in the lymphatic system of intestinal tract and taken up by Peyer’s patches. The developed systems specially lectinized liposomes followed by lectinized niosomes were found to preferentially accumulate in the mucosal and submucosal regions of the intestine and even penetrate the deeper layers indicating the role of ligand mediated interaction between carrier and the carbohydrate epitopes expressed by the biofilm infected intestine.

However, it must be stated at this point that the nature and type of biofilm may be associated with the same or different type of carbohydrate recognition domains and thus the uptake of ligand anchored systems depends upon the complexity and heterogeneity of the biofilm skeleton and glyco-calyx oligosaccharides. This fact should be considered while engineering the stable oral delivery systems for biofilm targeting.