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Biofilm Consortia on Biomedical and Biological Surfaces: Delivery and Targeting Strategies

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Microbial biofilms have been observed as congregates and attached communities on a diverse range of microecosystems of medicinal and industrial importance. Until recently, most investigations have been performed on planktonic (floating or fluid phase) microorganisms. After realization of the biofilm existence and their resilience toward conventionally adopted preventive strategies and antimicrobial agents, research has been shifted toward novel therapeutics based drug delivery and targeting approaches. With the emergence of various biofilm models and methods to assess biofilm formation and physiology, it is pivotal to discuss various novel strategies that may become the therapeutic tools and clinically adaptable strategies of the future. This review explores various novel research strategies studied to date for their potential in effective biofilm eradication.

KEY WORDS: biofilm; antimicrobial agent; resistance; drug delivery; drug targeting; ultrasound; liposomes; microspheres.

INTRODUCTION

Research issues have been redefined and revolutionized by the fact that most bacteria in the bio-environment aggregate as biofilms. This is a growth domain in which bacteria behave very differently compared to free-floating (fluid phase, planktonic) bacteria growing in laboratory cultures (1). Biofilms can be considered as microbial ecosystems representing different microbial strains and species in aggregation, which efficiently co-ordinate and co-operate to protect themselves against environmental stresses and facilitate the nutrient uptake for survival (2). They are layered aggregates and stable synergistic consortia of microorganisms attached to the surface of biomaterials and biological sites. The interaction that occurs between biofilms and their physical and chemical micro- and macro-environment, largely determines the extent and manner through which these bacterial communities cycle nutrients, degrade toxics, survive in hostile environments and resist conventionally administered antimicrobial agents.

BIOFILM ARCHITECTURE

Bacteria in a biofilm grow in matrix-enclosed microcolonies interspersed with variably dense regions of the matrix that include water and nutrient channels (3,4). The bacteria (microorganisms) adhere and remain immobilized in matrix of polymeric compounds, which are generally referred to as extracellular polymer substances (EPS). Typical constituents of EPS are polysaccharides (major) and proteins (minor) often accompanied by nucleic acid, lipids or humic substances. The bacteria in biofilms generally bind together in a sticky web of tangled polysaccharide fibres (known as slime substances of EPS) which connect cells and anchor them to a surface and to each other (Fig. 1). Within this microcosm, anaerobic and aerobic bacteria can thrive alongside each other, sharing water passageways and a complex structure. The polysaccharide coating is like a shielding coat and one or different types of bacteria collaborate to make an eventual bacterial biofilm (5). Biofilm bacteria have been shown to be morphologically and metabolically distinct from those growing in liquid cultures, which are also capable of forming biofilms, once they find a locus point to stick. The later is mostly provided by the bio-surfaces in different bioengineering, biotechnology and biomedical settings (5,6). The sticking to a bio-surface sets off a genetic cascade that turns on specific genes to express polysaccharides and/or to express surface receptors needed to establish the biofilm colonization.

BIOFILM RESISTANCE TO ANTIMICROBIAL AGENTS

The possible mechanism(s) of biofilm formation in an aqueous environment are exhaustively reviewed (7–10). Various microscopic and physical methods have been proposed and documented for use to assess biofilm formation and to study biofilm physiology and the possible role of various genetic and environmental factors in biofilm formation (7). The formation of an infectious biofilm on biomaterials appears to involve several mechanistic and sequential steps. The mechanism(s) are based on the initial microbial adhesion or attachment to a biological (or biomedical) surface followed by a cascade of events leading to the development of different
Fig. 1. Bacteria associated or attached as a biofilm with bio-surface (hypothetical)* A = Dense polysaccharide and epoxy-polysaccharide matrix, B = Microcosm and discrete micro-colonies of bacteria, C = Open water and nutrient channels, and D = Bio-surface to which the bacterial consortium is attached or adhered.

layers of biofilm micro-consortia. Figure 2 schematically presents the suggested mechanism, which is being extensively documented elsewhere (7–11).

However, the resistance of these biofilms to antimicrobial agents and/or biocides has been the major focus of research for the last few years. The relative resistance of microbial biofilms to antimicrobial agents has been accounted for due to transport-based and physiology-based mechanisms or a combination (11) (Table 1). Transport-based mechanisms indicate that the biofilms act as a barrier to antibiotic/antimicrobial diffusion (12). The main attributes of this mechanism rely on the features that govern transport rates and generate structural heterogeneity. External mass transfer resistance, which refers to the transport of a solute as it moves from the bulk fluid to the biofilm surface, further retards penetration. These solutes/materials can be soluble (microbial nutrients and organic solutes) or particulate (viable microorganisms, inorganic particles and antimicrobial agents). Structural heterogeneity is the most common feature of microbial biofilms (1–3). The biofilms are composed of different micro-colonies of bacterial cells in the bulk fluid, bulk fluid-biofilm interface and into the extreme interiors with different levels of dense exo-polymer matrix material and less dense water channels (5). These water channels transport oxygen (dissolved) to the biofilm, but limited diffusion and non-uniform oxygen use produce very low oxygen levels at the centers of cellular micro-colonies (13). This may explain the existence and even the physiological activity of fastidious anaerobes within mixed biofilms in an aerobic environment and further complicates the resistance to antimicrobial agents that are delivered and designed for single species based biofilms (3). The second explanation fo-
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* Compiled from (3,12-15).

...various types of biomaterial-centered infections in human (16). Fungal, protozoal and bacterial biofilms have been found on a variety of indwelling devices removed from patients with associated biomaterial-centered infections (17-19). The resistance of these biofilms to antibiotics and antimicrobial agents depends upon various factors. Not only do biofilms resist them, but also they are large enough to defeat the immune system. Biofilm bacteria have been protected from complement-mediated opsonic factors and phagocytic cells. The components of extra-cellular polymeric substances (EPS) of biofilm can also modulate the cellular immune responses (20). Consequently, infection of biomaterial implant may entail re-operation, osteomyelitis, amputation or may even lead to the death.

The properties of antimicrobial agents and antiseptics are clinically feasible for the prevention and treatment of plaque-related oral diseases. Many workers have reported the results of studies in which the minimum inhibitory concentrations of agents for cariogenic and periodontal-pathogenic bacteria have been determined. However, such data are relevant only to situations where the organisms of interest are in aqueous suspensions (fluid phase or planktonic), whereas in caries and the inflammatory periodontal diseases the target organisms are in the form of biofilms, a form in which they behave very differently (21). Recently, Stark and coworkers (22) revealed that Helicobacter pylori, a causative organism of gastric ulcer and associated gastric carcinoma accumulates at the air/liquid interface as water insoluble biofilms. The production of water insoluble biofilm by H. pylori may become an important parameter in elucidating resistance to host-defense factors and antibiotics. On the basis of micro-environmental pH homeostasis that regulates the growth and survival of H. pylori in vivo, a useful therapeutic and clinical strategy could be devised. The clinical efforts are focused on the development of preventive and therapeutic regimens to check the biofilm eliminating concentrations or biofilm killing concentrations of the antimicrobial agents in various conventional and local drug delivery devices (reviewed in 3). The following are the areas of biomedical and clinical sciences where the accumulation of biofilm needs special attention:

- Biomedical implants used for diagnostic and/or therapeutic procedures including cerebrospinal fluid shunts, orthopedic devices, artificial joints, wound drainage tubes, artificial hearts, prosthetic heart valves and cardiac pacemakers;
- Intravenous catheters especially continuous ambulatory peritoneal dialysis catheters;
- Contact lenses;
- Dental plaque mediated ailments (Caries and Periodontal pocket diseases); and
- Infected tissues of gastrointestinal tract, urinary tract, lungs, trachea and other organs.

Efforts and attempts are continuing to control and eradicate biofilms, using novel antibodies and the use of controlled and novel drug carriers. With the failure of conventional means to achieve therapeutic levels at the infectious sites of biofilm localization, either due to the ecological niche of the sites or the bacterial resistance toward the already existing therapeutic strategies, controlled and novel drug delivery strategies are appreciated.

**BIOFILM CENTERED INFECTIONS**

Biofilms are a major concern in the field of medical, pharmaceutical and biosciences especially in the case of a...
BIOFILMS AND BIO-PHYSICALLY MODULATED DRUG DELIVERY

Biophysical means have been developed and adopted to modulate the antimicrobial therapy over last few years. Electric fields and ultrasound applications have been used to enhance the efficacy of antibiotics/antimicrobial agents via two-fold action, i.e., biofilm penetration enhancement and killing bacteria through abrasive sterilization processes.

Ultrasound waves were investigated for biophysical modulation of drug release from delivery device into the bacterial biofilms. Ultrasound irradiation positively enhances the killing of Pseudomonas aeruginosa biofilms when combined with gentamycin by nearly two orders of magnitude (23). The effects of ultrasound frequency and duration on biofilm killing using antibiotics were optimized (24). The studies inferred that lower frequency of amplification produces higher levels of killing of bacterial biofilms of Escherichia coli, where the study on duration indicated that complete sterilization of a 14-h biofilm could be achieved after 6 h of exposure. In another study, Escherichia coli biofilms on polycarbonate disks were implanted subcutaneously into rabbits receiving gentamicin as a model antibiotic agent (25). Ultrasound was applied for 24 h and viable counts of the bacteria in the biofilm were made. Pulsed ultrasound significantly reduced bacterial viability to the excessively low level as compared against untreated biofilms.

Blenkinsop et al. (26) proposed the term “bioelectric effect” to describe that small “dc currents” could be used to enhance the efficacy of bioicides against Pseudomonas aeruginosa biofilms. Enhanced rate of biofilm elimination with antibiotic therapy was reported when a “dc current” was applied as part of the biofilm treatment (27). Photomechanical waves (PW) generated by ablation with high-pulsed lasers has been used as an extension to the electric field to synergies biofilm killing. While the heat and evaporation cause the ultrasound-assisted biofilm killing (28), the photomechanical waves-assisted effects were mainly attributed to the mechanical force (29).

Most of the studies have been focused on holding the electrical parameter (e.g., the dc current) constant and studying the result of varying biological variables (e.g., the level of bioicides). However, McLeod and co-workers (30) kept the biology (level of bioicides) constant and varied the applied electromagnetic field. These workers demonstrated a dose response curve for the current needed to produce increasing levels of killing of the bacteria in the Pseudomonas aeruginosa biofilm using tobramycin as a model antibiotic. Possible mechanisms for the bioelectric-effect-mediated antibiotic control of bacterial biofilms have been proposed (30). The enhanced activity was proposed to be due to an increased delivery of oxygen to the biofilms as oxygen is generated by in situ hydrolysis. It is possible that when oxygen levels reach toxic levels in the biofilm, it weakens the bacterial cells and subsequently renders them more susceptible to the antibiotics. In contrast, another possibility is that increased oxygen supply could enhance the growth within the depth of the biofilm, which would negate the reduced susceptibility of the bacteria in the biofilm due to their slow growth (31). Moreover, the active oxygen intermediates such as peroxides that are generated during the process may also cause a bioelectric effect (30).

In recent studies, Soukou et al. (32,33) reported the use of photomechanical waves for effective drug delivery to bacterial biofilms with their possible clinical adaptability. In their study, photomechanical waves were generated by ablation of a target with a Q-switched ruby laser and subsequently exposed to Actinomyces viscosus biofilms in the presence of methylene blue. These workers tested the hypothesis that photomechanical waves disintegrate and disorganize the structure of a microbial biofilm and thus increase the penetrability and permeability of simultaneously applied bioicides. Simultaneously administered methylene blue penetrated the biofilm population as recorded using confocal scanning laser microscopy. These studies revealed that a single photomechanical wave was sufficient to produce a 75% increase in the penetration depth of methylene blue into the biofilm. The enhanced permeability of biofilm population by photomechanical waves was considered as a therapeutic tool with potentials for photodynamic therapy using photodynamic compounds. These workers subsequently assessed the photodestruction of Actinomyces viscosus biofilms after their sensitization with methylene blue followed by irradiation with photomechanical waves and red light at 660 nm.

The approaches of using either ultrasound or electric waves including photomechanical waves may prove useful in delivering bioicides into biofilms of different species (caries, periodontitis, denture stomatitis, Helicobacter pylori infections of stomach, candidiasis) as well as in the treatment of prostatic medical device and contact lens-associated infections.

BIOFILMS AND LIPOSOMAL DRUG DELIVERY

Drug delivery and targeting to the bacterial biofilms has received current interests. However, the potential of drug delivery in the localization and/or targeting of biofilms still remain to be proved and adopted in the field of pharmaceutical research. The targeting could be realized using carriers, site-specific ligands and delivery of release modifiers. Targeting could be extended implicating intrinsic and inherent distribution profile of carrier (passive targeting). It can also be achieved using site-specific drug-carrier composite appended with suitable ligands to alter its distribution or uptake in the biological milieu and to release the drug in the proximity of bacterial biofilms (active targeting).

Among the various delivery systems directed against bacterial biofilm (mainly plaque or periodontal pocket flora, which represent the model biofilm in different studies) vesicular systems are found to be versatile in their disposition of the contained drug. These systems mimic the biomembrane in terms of structure and bio-behavior, and hence are investigated intensively for targeting bacterial biofilms.

Vesicular systems in general and liposomes in particular are the highly investigated delivery and targeting devices designed and developed for bacterial biofilm targeting (34, 44). Jones and Kaszuba (36) reported polyhydroxy-mediated interactions between liposomes constructed of phosphatidylcholine (PL) and bacterial biofilms. The targeting of liposomes to adsorbed films of bacteria was thought to be due to the interaction of the surface associated polymers of the bacterial “glyco-calyx” with polyhydroxy head groups of liposomal lipids. The theoretical basis of biofilm interactions of liposomes investigated by these workers was based on a three-
dimensional lattice model for the bacterial glyco-calyx and two-dimensional lattice for the liposome surface. The models were parameterized for the potential energy of interaction between surface of biofilm and liposome as a function of their separation. It was elucidated that a relatively small energy of interaction between the polyhydroxy-head groups (phosphatidylinositol) of the liposomal lipid and bacterium surface polymer residues (polyl phosphate polymers, teichoic acid) exists. This gives rise to a potential energy of interaction, which was found to exceed in excess than the classical double layer repulsive force and attractive dispersion force interactions. This potential energy of interaction exhibits quantitatively an energy minimum, which was found to be a function of the polyhydroxy-lipid concentration on the liposome surface. This model thus predicts an optimal liposomal composition for optimal adsorption of liposomes to bacterial biofilms. In accordance with the lattice model, these workers further demonstrated the adsorption of dipalmitoyl-phosphatidylycholine (DPPC)-PI liposomes to a range of biofilms of oral and skin-associated bacteria on solid-support, where optimum levels of PI for biofilm-adsorption were determined and reported.

These DPPC-PI based liposomes were further studied for biofilm targeting with encapsulated enzymes, glucose oxidase (GO) and horseradish peroxidase (HRP) (41). The systems were termed as "reactive liposomes". These reactive liposomes exhibited significant localization to biofilms (due mainly to PI component) and in the process released encapsulated enzymes in the close proximity of the biofilm. This subsequently led to inhibition of further bacterial growth as released enzymes in presence of their substrates, i.e., glucose and iodide, release species like hydrogen peroxide and oxo-acids, which are antibacterial in nature.

In different studies, however, various liposomal versions like cationic liposomes (39,40), lectinized or proteoliposomes (44,46), immunoliposomes (38,43) and liposomal hydrogels (47) were investigated for their targeting potential using various models of attached or aggregated bacteria (Table II). Sanderson et al. (39,40) reported adsorption of cationic liposomes over biofilms of skin-associated bacteria. Cationic liposomes (dipalmitoyl-phosphatidylcholine, cholesterol and stearylamine) were exposed to adsorbed biofilms of *Staphylococcus epidermidis* using a microtiter plate model (39). The interaction (as assessed by the apparent monolayer coverage of the biofilms by the liposomes) was described using Langmuir adsorption isotherm, which enabled the determination of maximum theoretical coverage of the bacterial surface and association/dissociation constants. Adsorption of SA-containing liposomes to biofilms is governed by several factors including hydrophobicity of bacterial strains, lipid compositions of liposomes, temperature and ionic strength of dispersion.

The results indicated that electrostatic effects mediate the attractive interaction between the cationic liposomes and negatively charged sites on the bacterial surface or the extracellular slime (e.g., teichoic acid). This was evident by two observations. Firstly, the increased ionic screening at higher ionic strength weakens the attractions between bacterium and vesicle (a decrease in dissociation constant) and secondly the compression of the diffused double layer surrounding these oppositely-charged surfaces leads to a decreased biofilm-vesicle dissociation constant. This subsequently results in a maximum theoretical coverage and hence enhanced population of liposomes gets attached to bacterial biofilm. Subsequent to surface attachment the release of encapsulated bioactive occurs within the vicinity of surrounding biofilms and thus a practical site-specific delivery could be negotiated.

To exploit the biofilm-associated surface determinants (antigens) for target selectivity, Robinson and co-workers (43) reported the specificity and affinity of immunoliposomes toward *Streptococcus oralis* biofilms using two different surface-bound monoclonal antibodies (anti-oralis antibodies 4718 and 4715) raised against antigenic determinants of the same bacteria. The anti-oralis immunoliposomes showed the greatest affinity and percent monolayer coverage when targeted to a range of different oral bacterial biofilms, i.e., *S. oralis, S. sanguis, S. gordonii, S. salivarius*, and *S. mutans*. The targeting affinity of immunoliposomes for *S. oralis* however was largely unaffected by the number of antibodies conjugated to the liposomal surface or by the net charge on the lipid bilayer. Moreover, anti-oralis immunoliposomes were relatively less specific for *S. oralis* than the free anti-oralis antibodies because of the non-specific interaction of the liposomes with other bacteria of typical multi-species biofilm.

Similar attempts were made to exploit the surface glycolconjugates/polyaspartic acid slime substances of the bacterial origin to target bacterial biofilm using lectin conjugated or

| Table II. Various Liposomal Systems Studied for Delivery of Bioactive to Bacterial Biofilms |
|----------------------------------------|------------------------|------------------|----------|
| Type of system | Composition | Bacteria (biofilm) | Ref. |
| PI-Liposomes | DPPC:PI | *Streptococcus mutans, S. epidermidis* | 36 |
| Cationic liposomes | DPPC:Chol:SA | *Staphylococcus epidermidis* | 40 |
| Reactive enzyme | DPPC:PI:GO:HRP | *Staphylococcus gordonii* | 41 |
| Cationic liposomes | DPPC:Chol:DDA | *Staphylococcus epidermidis* | 48 |
| Immunoliposomes | DPPC:PI:DPP:MB | *Streptococcus oralis* | 43 |
| Proteoliposomes | DPPC:PI:MBS | *Staphylococcus epidermidis, S. sanguis, Proteus vulgaris, S. mutans* | 46 |
| (sCon A) | DPPC:PLD:PI:MBS | *Staphylococcus epidermidis, Proteus vulgaris* | 46 |
| Protoliposomes (WGA) | DPPC:PL:PS:DP-DSPE | *Pseudomonas aeruginosa* | 47 |

Abbreviations: DPPC = dipalmitoyl-phosphatidylcholine, DPPG = dipalmitoyl-phosphatidylglycerol, PI = phosphatidylinoitol, Chol = cholesterol, SA = stearylamine, GO = glucose oxidase; HRP = horseradish peroxidase, DDA = dimethyldioctadecylammonium bromide, DPP = dipalmitoyl-phosphatidylethanolamine, MBS = s-maleimidobenzo-2-nitro-4-hydroxysalicylicacid, PL-DSP = PL-Diacontoyl-phosphatidylethanolamine, sCon A = s-concanavalin A, WGA = wheat germ agglutinin.
anchored liposomes. In most of these studies, N-succinimidyl-S-acetylthioacetate (SATA) derivatives of the lectins were conjugated through the reactive m-maleimidobenzoxyl-N-hydroxysuccinimide (MBS) derivative of dipalmitoyl phosphatidylethanolamine (DPPE). This lipid derivative was then incorporated to liposomes of DPPC (or DPPG) and PI by vesicle extrusion technique. Succinylated Con A (sCon-A)-bearing liposomes (proteoliposomes) have been found to be effective for the delivery of Trichosan to biofilms of skin-associated bacteria, Staphylococcus epidermidis and Proteus vulgaris, and the oral bacteria Streptococcus sanguis (48). Even on exposure to a very short time the succinylated Con A bearing liposomes were retained by the bacteria biofilm and eventually delivered Trichosan in the cellular interiors of biofilms. The targeting was assessed by an apparent monolayer coverage (%-samt) of the biofilms by liposomes and the optimum levels of phosphatidylinositol and Concanavalin-A were established using a biofilm model grown on microtiter plates. In contrast, inhibition or cell death rates for free Trichosan under the same experimental conditions following the exposure to the periodontal pocket bacteria was significantly less. The same group of workers (50) compared the role of surface bound lectins (sucinylated Con-A and Wheat germ agglutinin, WGA) for their sensitivities toward various oral and skin-associated bacteria. The oral bacteria Streptococcus mutans and S. gordonii and the skin associated bacteria Corynebacterium bovis were successfully targeted using succinylated Con-A bearing proteoliposomes while the skin associated bacterium Staphylococcus epidermidis was targeted with WGA bearing proteoliposomes. In these experiments, both cationic and anionic as well as proteoliposomes were compared for their relative efficiency in delivering the bactericide Trichosan to biofilms. The concept of lectin-carbohydrate interaction accentuates the potential of lectin bearing liposomes as targeted delivery device for the control of dental plaque and gingivitis as established by Jones and co-workers (35,37,45). In different studies, a “lectin-target enhancement” factor (LTE, lectin liposomes binding per mole of lipid/naked liposome binding per mole of lipid) was established and used as an indicator of targeting efficiency of different liposomal systems. The LTE was measured in terms of binding of liposomes to the target site either radiochemically using an appropriate labeled phospholipid or by inhibition of an appropriate ELISA using an antibody, which is specific for the target surface.

To further optimize the targeting as a function of mole percent of cationic and anionic lipids, Jones and associates (42) prepared liposomes of dipalmitoylphosphatidylcholine (DPPC) incorporating the cationic lipids stearylamine (SA), dimethyl-dioctadecyl-ammonium bromide (DDAB) and dimethylammonioethane carbamoyl cholesterol (DCChol) and the anionic lipids dipalmitoylphosphatidylethanolamine (DPPG) and phosphatidylglycerol (PG). The delivery of oil-soluble bactericide Trichosan and the water-soluble bactericide chlorhexidine was studied for a number of liposomal compositions. Targeting was recorded to be most effective for DPPC-cholesteryl SA (for both bactericides), DPPC-DPPG and DPPC-PI based liposomes (for Trichosan). These systems were studied on S. epidermidis and S. sanguis biofilms. Double labeling experiments using [14C]-chlorhexidine and [3H]-DPPC suggested that there was an exchange between adsorbed liposomes, which as a result delivered bactericide to the biofilm and those in the bulk solution implying a diffusion mechanism for bactericidal delivery.

Recently the potential of ligand-mediated biofilm targeting has been explored using liposomes anchored with suitable site-directing ligands. Vyas and co-workers (51,52) proposed lectin-carbohydrate interaction as principle mechanism for the delivery of metronidazole against the bacterial flora of the periodontal pocket. Various engineered liposomes, i.e., mannan (polysaccharide) coated, sialo-mannan coated and lectinized (Con-A), were studied for their interaction with surface epitopes expressed on bacterial cell surface as glycoalexins. The targeting potential of these systems was expressed as % biofilm growth inhibition using a microtiter plate model for bacterial infection. Surface engineered liposomes provided excellent biofilm growth inhibition as compared against their plain counterparts however the mechanisms of their actions are yet to be fully elucidated.

Even though these developed liposomes are functional in vitro against bacterial biofilms, there are some problems associated with binding liposomes to the surface of medical devices. If drug loaded liposomes occupy all surface area, insufficient drug would be released to prevent bacterial adhesion for a significant period. In addition, the shear forces generated during the handling and insertion process would displace liposomes from the surface. DiTirro and co-workers (47) reported an approach to control bacterial biofilm formation on urinary catheters by sequestering the drug-loaded liposomes within a biocompatible matrix located on the surface of the catheter. The system consisted of a poly (ethylene glycol)-gelatin hydrogel in which liposomes consisted of DPPC and PEG-diastearoylphosphatidylethanolamine (PEGDSPE) were sequestered. The three-dimensional gel matrix is capable of accommodating large quantities of drug-loaded liposomes, while simultaneously protecting the liposomes from membrane disrupting shear forces encountered during handling and insertion of the device. The prolonged release pattern of ciprofloxacin from these liposomal hydrogel preparations was approximated by zero-order release kinetics. Liposomal hydrogel coated catheters were also tested against Pseudomonas aeruginosa biofilms in terms of percent inhibition of bacterial growth and percent reduction in bacterial adhesion to treated catheter surfaces. It could be inferred that the zones of inhibition created by the ciprofloxacin loaded liposomal hydrogel preparations were approximately five-fold larger than the inhibition zones of control catheter sections treated with drug only. Similarly, hydrogel coating was found effective in preventing biofilm cells from adhering to the catheter surface as no bacterial cell viability was detected on these surfaces during a seven day treatment.

**BIOFILMS AND MICRO-PARTICULATE DRUG DELIVERY**

Biodegradable polymers for localized delivery of antibiotics have emerged as an important approach for treating biofilm infections associated with medically implanted devices. Several studies reported the microspheres prepared from biodegradable polymers and loaded with suitable antibiotics against in vitro developed biofilms. The relative effectiveness of poly (L-lactic acid) microspheres loaded with ciprofloxacin hydrochloride was investigated against periodontal implanted biofilms of Pseudomonas aeruginosa in a rub-
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The viable counts of *Pseudomonas aeruginosa* were markedly reduced or eliminated from the catheter, the device and the peritoneal wall in microsphere-treated rabbits as compared against rabbits treated with free drug. The viable counts were made from histological and scanning microscopic observations. A sustained release profile of antibiotics was recorded from a matrix type biodegradable device. The same group of workers investigated the need to maintain a sustained drug concentration above the biofilm eradication concentration to obliturate aged biofilms of *Pseudomonas aeruginosa* and *Staphylococcus aureus* (54). In the specially developed modified open in *vitro* chemostat system, the drug was continually diluted at the site of administration (peritoneal cavity). The kinetics of release of ciprofloxacin as a function of drug loading and the dose of microspheres were correlated with the rate and extent of killing and eradication of the planktonic cells and aged biofilm cells cultured on pieces of silicone tubing in the chemostat. A correlation was established between sustained ciprofloxacin concentrations and the eradication of biofilms from both *Pseudomonas aeruginosa* and *Staphylococcus aureus*.

Habib and coworkers (55) used the microspheres for biofilm eradication to treat bone-associated bacterial biofilm infections, osteomyelitis. The microspheres of poly (glycolic acid-co-ε-DL-lactide) loaded with ofloxacin provided a biphasic release profile with an initial fast release followed by a slow release phase, quite typical of a sustained release formulation. This release pattern was found to be pivotal for biofilm eradication in various studies carried out by these workers (53,54).

**FUTURE PERSPECTIVES**

The invention of biofilms as the target for bioicides has revolutionized the approach of research in medical, pharmaceutical, and biosciences. A recent innovation in this regard is the search of biomaterials that resist bacterial congregation. Many new devices have been introduced, for example, with hydrophilic outer layers, antimicrobial coated surface, low-surface energy and carbon-rich materials, highly bioincompatible substances, biodegradable materials, and cell or protein grafted surfaces (56-57). The use of phosphorylcholine (PC) surface allograft materials improves the biocompatibility of medical devices in contact with blood or tissue as it produces a non-thrombogenic surface (58). Recent experimental studies supported the effectiveness of PC-grafted or surface medical devices to retard bacterial and crystal adhesion (biofilm formation) from the biological fluids (59). Not only the advances in polymer-grafted biomaterials but also the biofilm surface determinants (60-61) and genetic factors responsible for biofilm formation (62) are going to dominate the biofilm research of future.

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

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Liposomes constructed of egg phosphatidylcholine (EPC), cholesterol (Chol) and stearylamine (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 (% BCI). 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-enlyx’ of bacterial biofilm.

1. Introduction

Liposomes can be used as carrier systems for the presentation and transportation of various bioactive 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 system 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 metobetical 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 (LD50) and high efficacy against gram negative bacteria (MIC8 = 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 intrapocket bacterial 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 the interaction with sugars expressed on cell surface glycoconjugates (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 (stearoylamide 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 stearoylamide (+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/v to total lipids. Further increasing the concentration of Con-A to a total lipid weight ratio of 0.1:1 w/v results in a negative Zeta potential (~ -5 ± 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 stearoylamide. 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 deluging 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 ± 0.8% to 22.8 ± 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 calcium 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 nM (A and C) and 10 nM (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) 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

<table>
<thead>
<tr>
<th>Composition</th>
<th>Lecin to total lipid ratio</th>
<th>Vesicle size (Avg)</th>
<th>% Entrapment</th>
<th>Surface charge</th>
</tr>
</thead>
<tbody>
<tr>
<td>PC: Chol: SA</td>
<td>-</td>
<td>2.8 ± 1.2</td>
<td>27.1 ± 0.8</td>
<td>+ (35.7 ± 2.1)</td>
</tr>
<tr>
<td>(PC: Chol: SA: Con-A)</td>
<td>0.001:1</td>
<td>2.9 ± 0.9</td>
<td>22.4 ± 0.6</td>
<td>+ (14.9 ± 0.9)</td>
</tr>
<tr>
<td>(PC: Chol: SA: Con-A)</td>
<td>0.01:1</td>
<td>2.9 ± 0.9</td>
<td>22.4 ± 0.6</td>
<td>+ (2.7 ± 0.5)</td>
</tr>
<tr>
<td>(PC: Chol: SA: Con-A)</td>
<td>0.1:1</td>
<td>2.9 ± 1.1</td>
<td>22.8 ± 0.8</td>
<td>- (5.0 ± 2.0)</td>
</tr>
<tr>
<td>(PC: Chol: SA: Con-A)</td>
<td>1:1</td>
<td>3.2 ± 0.1</td>
<td>22.8 ± 0.8</td>
<td>- (27.6 ± 3.4)</td>
</tr>
</tbody>
</table>

* The liposome compositions are based on a molar ratio of PC: Chol: SA (1:1:0.1)
* The ratio of lectin to total lipid is presented on a weight basis
* Average vesicle size of 93% of the population was obtained using phase contrast microscope coupled with calibrated eyepiece (n = 6)
* % Entrapment was determined using classical dialysis method (n = 6)
* Surface potential of the drug loaded lectinized liposomes at different weight ratio of lectin to total lipid (n = 3)
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:

\[
\% \text{Con-A latency} = \left( \frac{[\text{Con} - \text{A}]}{[\text{Phospholipid}] \text{at the start of stability study}} - \frac{[\text{Con} - \text{A}]}{[\text{Phospholipid}] \text{at an appropriate incubation time}} \right) \times 100
\]

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

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

<table>
<thead>
<tr>
<th>Formulations</th>
<th>% BSM interaction at different mM Ca²⁺</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1 mM</td>
</tr>
<tr>
<td>Before adding competing sugar¹</td>
<td></td>
</tr>
<tr>
<td>Drug loaded (PC) liposomes</td>
<td>2.1 ± 0.1</td>
</tr>
<tr>
<td>Lectinized (PC) liposomes</td>
<td>7.6 ± 1.2</td>
</tr>
<tr>
<td>After adding competing sugar²</td>
<td></td>
</tr>
<tr>
<td>Drug loaded (PC) liposomes</td>
<td>1.9 ± 0.6</td>
</tr>
<tr>
<td>Lectinized (PC) liposomes</td>
<td>1.6 ± 0.2</td>
</tr>
</tbody>
</table>

¹ Competing sugar used was α-methyl mannoside (MM)
² All experiments were run in triplicate

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 Ca²⁺. 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.

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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 nm) of a given test mixture against that of tubes containing S. mutans alone and can be expressed as given below:

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

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 (MICs) 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
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 mannosyl-residues of the bacterial surface glycoalyx. The lectin-carbohydrate interaction could lead to loci 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 key 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 *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 (~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 antimicrobial 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 glyco-conjugates 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 mannosyl 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 Sigmes (St. Louis, MO, USA) and used as supplied. Cholesterol (Chol), diethyl phosphate (DCP), stearoylamine (SA) and subsidiary mucin (BSM) were from Fluka chemicals, Sigma-Aldrich, Comauvalin A (Con-A) from Comauvalin, and 1,2-propanediol was from Sigma (St. Louis, MO, USA). All other chemicals used were of analytical grade, unless otherwise mentioned and were obtained from Qualigens (Ghaziabad Ltd., India). Streptococcus mutans bacteriophage PR-1034 was obtained from the Institute of Microbial Technology (IMTEK, Chandigarh, India) as bacterial strains. The metronidazole MIC was determined by the dilution susceptibility test as described by Baker et al. [29]. Metronidazole MIC₅₀ for Streptococcus mutans was recorded to be 0.18 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/unseeded) liposomes were prepared by first removing the solvent using a rotary flash evaporator from a 250 µmol mixture of EPC, Chol and charge interrupting conjugates to form a thin film on the inner wall of a round bottom flask. The cast 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 a lipid dispersion off 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 lipids. These drug loaded plain liposomes were centrifuged thrice (10,000 g) for 60 min to remove any unentrapped metronidazole. The liposomal population was harvested and resuspended for vesicle sizes and those above 1 µm (as retained overzoned on Aerodiscs, 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 with the oppositely charged lipid bilayer. The cationic behavior of concavalin A was exploited. Lectin acquires a different charges at pH values above and below and in the vicinity of its isoelectric point [17]. To the whole vesicular dispersion obtained (total lipid is 7.2 × 10⁻¹⁳ mol² m⁻²) 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 stearylamine based formulations and to pH 6.0 in the case of dicetyl phosphate based formulations using mixed phosphate buffers. The dispersion obtained were kept incubated for an optimized period of 4 h. Charge induced coating of Con-A over liposomal surface was further assessed using a Neophotometer (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 liposomal dispersions 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, the mobility of liposomal dispersion in PBS (pH 7.4, 0.001 M) using the Zeta-potentiometer [30]. Unconjugated Con-A from liposomes conjugated Con-A was separated by centrifugation at 100,000 g for 30 min and experiment was repeated for 7 cycles. Aliquots of the clear supernatant 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 in 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; Hitachi, Japan). 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, Bismark, Germany). Vesicle dispersions were appropriately diluted and wet mounted on a hemocytometer and photographed through a microscope. The negatives were processed prior to print on a color film paper using an enlarger with an adjusted magnification × 1 (120). Diameter of around 500 vesicles were noted for each sample and the average vesicle size was calculated.

The % entrapped was determined and expressed as the percentage of added drug entrapped 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. Predicted vesicular dispersions were centrifuged at 100,000 g for 60 min. The pellets that obtained were resuspended in 0.01 M PBS (pH 7.4) and the process repeated 3 times. Vesicles were lyzed by adding 1.0 mol of 0.14% w/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 234 nm [31]. Liposomes were assumed in terms of total lipid phosphorus. The concentration of phosphorus in the liposomes was determined by measuring inorganic phosphate after acid hydrolysis at 180 °C in 70% HClO₄ [32]. % Entrapment was expressed as % entrapment/mg of lipd.

4.5. In vitro ligand affinity and activity studies

The activity of liposome coated Con-A towards experimentally provided bovine serum albumin (BSA) major substrate marker was evaluated by measuring the release of BSA from the model substrate marker with different sugars were studied [33]. BSA is a glycoprotein consisting of six different sugars namely, N-acetylglucosamine (168.0 µg/mg BSM), N-acetyllactosamine (60.2 µg/mg BSM), galactose (52.2 µg/mg BSM), mannose (20.7 µg/mg BSM), fucose (9.5 µg/mg BSM) and saccharic acid (16.9 µg/mg BSM). Different concentrations of Ca²⁺ were added to the reaction mixture to activate binding specificity of immobilized lectin with carbohydrate [34]. The in vitro biological activity of the liposomal system was determined by mixing 1.0 ml of BSA solution with 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 (20 mM) as a competing sugar in the BSA bulk solutions. % BSA 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 conducted for 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 gingivitis status. The stability of the lectinized liposomes was 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 pepsin per 10 ml, 2 g yeast extract, 5 g/l proteose 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 conducted by incubating 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 the free and bound lectin. The Con-A affinity per mole lipid was calculated and expressed as the percentage of the same remaining associated with liposomes. The Con-A was estimated using the 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 vivo targeting elucidation

In vivo targeting studies were performed on axenically grown broth culture of Streptococcus mutans. % Bioadhesion (BA) was the parameter studied. Streptococcus mutans bacterial strains 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 inoculated 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 laminar flow bottles at 37 °C for 24 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 washing process was repeated twice 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 predominately present in periodontal pocket infections. Brain heart infusion broth (4.5 ml) was inoculated with 2.5 × 10⁸ colony-forming units (CFU) of S. mutans, obtained from an early stationary phase of the bacterial biofilm 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 spectropho...
photonometrically 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 (MICx2) was added from the metronidazole bearing plain and leucitoned liposomes. After incubation of a bacteria-vehicle mixture for the scored period of time, growth inhibition was measured periodically for different formulations. The growth was recorded by measuring optical density (550 nm) of the dispersions using a Shimadzu 1601A UV/VIS DB spectrophotometer (26).

4.8. Statistical analysis

The anti-microbial activity of metronidazole loaded plain and lecuitened liposomes against S. marcescens 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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Polysaccharide coated niosomes for oral drug delivery: formulation and in vitro stability studies

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Non-ionic surfactant vesicles (niosomes) were prepared and appended with a polysaccharide cap using hydrophobic anchors. Hydrophobilized polysaccharides, O-palmitoyl pullulan (OPPs) and cholesterol pullulan (CHPs) were anchored onto propranolol · HCl containing preformed niosomes. The coated niosomes were characterized for average vesicle size, size distribution, shape, encapsulation efficiency and in vitro release profile and were compared with their uncoated counterparts. No significant difference was observed in % encapsulation (P > 0.05 in a rank sum test) of polysaccharide coated and uncoated vesicles. In vitro release studies however, revealed a significant lowering (P < 0.01) of drug release for the coated systems in simulated gastric and intestinal fluids with a biphasic release profile. The influence of the hydrophobilized polysaccharide cap on niosomal membrane integrity and stabilization against harsh bio-environment conditions was also investigated. The parameters investigated include detergent and bile (bile salts and fresh-pooled rat bile) challenge, freeze-thaw cycling, osmotic stress, and long term and shelf stability studies. It was seen that at higher bile salt concentrations and detergent content, uncoated niosomes underwent bilayer solubilization into intermediate micellar structures, whereas coated niosomes were able to maintain their structural integrity as reflected from their higher % latency for the entrapped water soluble agent. Similarly, freeze-thaw cycling could not bring any fusion or collapse of the niosomal membrane (unlike uncoated ones). Furthermore, the exceptional shelf stability of the coated vesicles both at 37 ± 1 °C and at 4 ± 1 °C establishes the potential of polysaccharide-coated niosomes as an oral delivery system for water-soluble agents. Results from OPPs and CHPs coated niosomal systems for their oral stability potential are compared.

1. Introduction

Nonionic surfactant based vesicles (niosomes) are self-assemblages of non-ionic amphiphiles into closed bilayer structures. Their low costs, greater stability and resultant ease of storage [1] has led to the exploitation of these vesicles as alternatives to phospholipid-based vesicles, mimicking most of the inherent characteristics of lipid vesicles. Further, they are chemically and mechanically more stable and osmotically active as compared against vesicles from natural (phospholipid-based) origin. Hydrated bilayer vesicles however, are not deemed to be thermodynamically stable and are thought to represent a metastable state in that the vesicles possess an excess of energy [2]. Furthermore, the method of formulation [3], the nature of nonionic surfactants [4] and encapsulated drug/macromolecules [5] were found to influence membrane fluidity/rigidity and permeability characteristics. The leaching of hydrophilic drugs from the aqueous domains of the niosomal bilayers upon storage is an area of considerable interest. The temperature of storage of these dispersions must be strictly controlled. A wide variability in storage temperature of the system often leads to a change in the fundamental nature of the system [6]. Furthermore, high concentrations of detergents or soluble surfactants are incompatible with niosomal systems and cause eventual solubilization of the vesicles to form mixed micelles and a host of intermediate aggregates [7–9]. These vesicles are thus predicted to transform into bilayer stacks against the challenges of physicochemical and bio-environment stimuli. To produce a system with optimal stability requires to slow down these transformations resulting in a product with a reasonable shelf life. Methods to enhance the stability of niosomes are abounding in the literature [9–12]. The inclusion of a charged molecule in the bilayer alters the electrophotorelectric mobility and makes it positive with the inclusion of stearylamine or negative with dicetyl phosphate, thus preventing niosomal fusion/swelling or aggre-
2.1. Characterization of hydrophobized polysaccharides (HP)

Pullulan is a linear α-glucan, produced by the yeast-like fungus *Pullularia pullulans*, in which about 480 maltotriose units are linked by α(1 → 6) glycosidic bonds. Unlike other naturally occurring polysaccharides, pullulan is known to protect plasma membranes against physico-chemical stimuli, such as osmotic pressure and ionic strength. However, when adsorbed onto the vesicle surface, it is easily desorbed on dilution or mechanical agitation [16]. However, chemically modified pullulan (hydrophobized pullulan) was found to strongly interact with the vesicles by inter-digitizing the hydrophobic legs of pullulan derivatives into the outer half of the bilayer [13]. In this study, pullulan was chemically modified with O-palmitoyl and cholesterolyl anchors using the procedures of Hammerling and Westpal [17] and Sato [18] with minor modifications. Pullulan was partially hydrophobized by palmitoylation (OPPu) or otherwise by cholesterol esterification (CHPu) (Schemes 1 and 2). Pullulan derivatives get inserted/inter-digitized into the bilayer on incubation via hydrophobic anchors, extending the polysaccharide portion into the aqueous milieu. The derivatization products were characterized by IR- and ¹H NMR spectra. The derivatization procedures employed to prepare OPPu and CHPu result in a degree of substitution of 1.57 palmitoyl chains and 0.71 cholesterol molecules per hundred saccharide units of pullulan as estimated by ¹H NMR analysis. Hydrophobized pullulan was also characterized by IR spectroscopy to identify carbonyl groups, and thus, to ascertain that pullulan is covalently bound to palmitoyl or cholesterol anchors. A frequency shift of most characteristic C=O stretching vibration (original band at 1735 cm⁻¹) was found. This could be ascribed to a consequence of intramolecular hydrogen bonds between carbonyl and hydroxyl groups that lower the stretching force vibration of the C=O band (1645 cm⁻¹ for OPPu and 1690 cm⁻¹ for CHPu). These observations are suggestive of an ester bond between pullulan and hydrophobic moieties indicating that they are not just physically admixed.

Scheme 1

![Scheme 1](image)

**Abbreviations**: SMCA = Sodium monochloracetate, EDMA = Ethylene diamine, CDI = 1-Ethyl-3-(3-dimethylamino)propylcarbodiimide, CHOL = Cholesterol, DMF = Dimethyl formamide, DMSO = Dimethyl sulfoxide

2.2. Characterization of polysaccharide coated niosomes

An artificial cell wall consisting of hydrophobic palmitoyl and cholesterol anchors was assembled on the outer surface of the niosomes. When added to niosomes the hydrophobic anchors interact with the outer half of the bilayer orienting and projecting the hydrophobic portion towards the aqueous bulk. In this way a two dimensional network of polymers is framed around the niosomal membranes (Scheme 3). The conductivity of the vesicles (in micromhos) was measured until stabilized to ensure completion of the coating (Systronics Conductivity Bridge, 305, India). Simultaneously, conductimetric measurements were made to optimize the hydrophobized polysaccharide to surfactant ratio and incubation period of pullulan conjugation to vesicles. The results suggest that at an applied voltage of 10 mV, the conductances of the vesicles stabilized at a lower ratio of CHPu to surfactant (0.01:1) than OPP to surfactant (0.1:1). This could be due to the fact that CHPu could have filled the loci of defects by becoming a part of the niosomal membrane, thus establishing an equilibrium at lower concentrations of coat material. Six hours were found to be the optimum time for coating of the niosomal bilayer, as no appreciable changes were recorded in the conductance of the coated systems at 10 mV beyond this time (data not shown). Niosomes derivatized with hydrophobized pullulan were subjected to vesicle type, shape and size analysis with the help of phase contrast microscopy (Leitz, Biomed, Germany) (Table 1). The niosomal population was previously harvested and screened for vesicle sizes and those above 0.88 (as retained oversized on ashless hardened Whatman paper, 0.88 μm) were taken for coating and subsequent stability protocols. Vesicles were found to be spherical and multi-lamellar with a size range of 2.5 μm to 5.5 μm and a mean vesicle diameter of 3.5 ± 0.75 μm for OPPu coated and 3.7 ± 0.9 μm for CHPu coated niosomes. Opacity of the system with the extinction of bitiranges revealed that coated vesicles were relatively larger in mean size than plain niosomes (uncoated). This may be accounted to the polymeric coating, a dual diffusion barrier on the niosomal surface.

Encapsulation efficiency of drug in CHPu and OPPu compositions were 27.5 ± 0.75 and 30.1 ± 0.7, respectively.

Scheme 3

![Scheme 3](image)
Table 1: Composition and characterization of various niosomal systems

<table>
<thead>
<tr>
<th>Composition</th>
<th>Molar ratio</th>
<th>Shape/phase contrast microscopy</th>
<th>Average vesicle size</th>
<th>Encapsulation efficiency</th>
</tr>
</thead>
<tbody>
<tr>
<td>Span 60:CH</td>
<td>(7:3)</td>
<td>Multilamellar, birefringence visible</td>
<td>2.7 ± 1.2</td>
<td>32.7 ± 1.2</td>
</tr>
<tr>
<td>Span 60:CH:DCP</td>
<td>(7:3:0.5)</td>
<td>Multilamellar, birefringence visible</td>
<td>2.9 ± 1.2</td>
<td>35.5 ± 0.8</td>
</tr>
<tr>
<td>(Span 60:CH:DCP):CHPp</td>
<td>(7:3:0.5:0.1)</td>
<td>Lamellarity indistinguishable, opaque appearance</td>
<td>3.7 ± 0.9</td>
<td>27.8 ± 0.6</td>
</tr>
<tr>
<td>(Span 60:CH:DCP):OPPp</td>
<td>(7:3:0.5:0.1)</td>
<td>Lamellarity indistinguishable, opaque appearance</td>
<td>3.5 ± 0.75</td>
<td>30.4 ± 0.6</td>
</tr>
</tbody>
</table>

revealing that CHPp and OPPp coatings did not affect significantly the initial levels of encapsulation (35.5 ± 0.1 recorded for plain vesicles). Further, the decrease in encapsulation efficiency was statistically insignificant when compared (P > 0.05) in a rank sum test manner. The niosomal coating with hydrophobized polysaccharide (HP) however retarded the leaching of entrapped drug in simulated gastric (SG) and simulated intestinal (SI) fluids. Moreover, the leaching profile remained the same as in either case it followed biphasic release kinetics. Fig. 1 presents % drug leached as a function of time recorded over various time intervals (1, 6, and 12 h). It could be seen that CHPp coated niosomes were able to retain 90.5 ± 0.7% of the drug, while OPPp coated niosomes showed 82.9 ± 0.1 drug latency at the end of the experiment. As the incubation periods increased the drug release also increased in the case of plain niosomes for a longer time period and an almost sustained release profile was observed near the end of the experimental protocol. For the coated systems, however, a biphasic release was observed. After the initial rapid release, a sustained and slower second phase was observed and maintained throughout the study protocol. However, the extent of drug release was comparatively higher from the OPPp coated niosomes than in CHPp coated niosomes. The results recorded in terms of drug leaching are significantly better than that of the plain niosomal system which could retain 32.5 ± 0.5% of the drug under the same experimental conditions. The drug leaching profiles followed more or less the same trend in the SI fluids (Fig. 2), however the relative and overall drug leaching was slightly higher (as compared to SGF) in the case of polymer coated vesicles (17.6 ± 0.12 and 25 ± 0.1, respectively, from OPPp and CHPp coated niosomes). The leaching recorded was significantly low (P < 0.01) as compared to plain niosomes (nearly 65.1 ± 0.4). A comparatively faster and higher release in the case of CHPp coated niosomes (reverse to the SG fluid) could be ascribed to the leaching of the intervening cholesterol anchor (Fig. 2). The low release (leaching) rates (less than 25% over a period of 12 h) may prove worthwhile for the therapeutic systems where the stability of the encapsulated contents and the integrity of carrier system are desirable.

To gain more information about the interaction of the delivery systems in the harsh environments encountered in biological milieu, polysaccharide coated niosomes were subjected to various stability studies.

2.3. Influence of hydrophobized pullulan anchors on membrane stability

*In vitro* stability studies revealed that coating of the outermost surface of niosomes with a naturally occurring polysaccharide bearing hydrophobic substituents (capable anchoring into the bilayer membrane) imparts stability to the niosomes. The first apparent effect of webbing hydrophobized pullulan onto the niosomal membrane was the protection against bile salt/detergent solubilization of the system and encapsulated drug. Stability of coated niosomes were tested by incubating the dispersion with different mM concentrations of bile salts (below, above and in the vicinity of CMC) and with fresh rat bile at 37 ± 1°C for a period of 6 h. In all stability experiments with different bile salts and at various concentrations, no significant changes in vesicle size, integrity and drug content were recorded. The same trends hold for the incubation studies with pooled rat bile. It can be seen from Fig. 3 that at salt concentrations below and at the CMC the drug content was not affected even in the case of plain niosomes, but at higher concentrations (above CMC) an apparent decrease in the % of initial content was recorded. However, the coated niosomes were shown to be tough and resistant...
against detergent challenge, a loss of nearly 10 \pm 5\% of initial drug content that was recorded could be ascribed to the normal course of drug release pattern from the heterogeneous dispersion. The solubilization of bilayer via the build up of CMC of glycodeoxycholate molecules within the niosomal membrane, followed by micellization could be proposed as the possible mechanism for decreased residual drug content at higher bile salt concentrations (for plain niosomes) [19, 20]. Dual diffusion barrier of coated niosomes was instrumental in its stability against the bile salts and fresh rat bile.

In a parallel experiment the relative stability of the coated niosomes was measured in terms of % drug leaching against the challenges of detergent, decyl-PEG. External drug was removed by chromatography of niosomes over Sephadex G-50 prior to the experiment. Addition of the detergent decyl-PEG-300 caused a rapid release of entrapped drug, indicating the structural defects in the niosomal membrane. The maximum drug level (100%) was obtained after the addition of 0.1% w/v Triton X-100. It could be seen that the drug leaching was stabilized at a hydrophobized pullulan to surfactant ratio of 0.01:1, far below the optimized ratio at which the bilayer membrane gets rigidified. The same trends have been followed irrespective of the incubation time (1 and after 6 h) with negligible variation in leaching. These results are in accordance with the membrane solubilization reported by Uchehg and Vyas [9]. High concentrations of detergents are incompatible and cause eventual solubilization of the vesicles to form mixed micelles and a host of intermediates. Coating the niosomes with hydrophobized pullulan however severely retarded the rate of detergent induced release (Fig. 4). Relative protection against detergent degradation started at slightly lower HP/surfactant ratios than those which decrease the fluidity of the membrane. Prolonged incubation with HP did not influence the stability of the niosomes.

Freezing of niosomes in liquid nitrogen, and subsequent thawing at room temperature, normally leads to a collapse of the niosomes and induce fusion of the membranes due to dehydration [22]. Consequently, freeze thawing of niosomes results in a nearly complete release of the encapsulated contents. Fig. 5 is the graphical presentation of the results of the freeze thawing studies of the polymer-capped niosomes. It could be seen that the drug leaching was stabilized at a hydrophobized pullulan to surfactant ratio of 0.1:1, an optimum ratio at which the bilayer membrane gets rigidized. The plain vesicles lost almost 71.5 \pm 2\% of drug in the experimental time (not shown), whereas the coated vesicles lost only 15 \pm 5\% in the same period of stress studies. The uncoated niosomes lost their contents after the stress challenge (100\% value). At the optimum HP concentration 90–92 \pm 1.7\% the niosomal population was observed to be intact, whereas the same concentration of unmodified pullulan (associated to niosomal membrane by adsorption) could not offer an appreciable protection to niosomes (not shown). For the earlier mentioned reasons, cholesterolated pullulan was found to be better in niosomal membrane stabilization. The vesicle integrity of hydrophobized polysaccharide coated niosomes was assessed following osmotic stress under increasing concentrations of different molar solutions of sodium chloride. Pullulan capped vesicles were found to resist the tonicity gradience. As evident from Fig. 6, the average vesicle size was found to increased in hypotonic environment, whilst under hypertonic conditions a marginal shrinkage and resultant decrease in the average size was observed. In hypotonic environment (50 mM and 100 mM) the plain vesicles show an increase in size, whereas under hypertonic conditions (200 mM and 250 mM) the vesicles shrink due to the osmotic variation.
in the surrounding fluids. It is evident that except of a marginal increase in response to the hypotonic media, no appreciable change has occurred in the case of polymer capped systems. Insignificant changes in vesicle size of the pullulan coated niosomes, reflected the better vesicle integrity offered by the polysaccharide cap against the osmotic challenges.

Long term stability of the niosomes was examined by measuring the spontaneous release of encapsulated contents as a function of time at ambient temperature. It could be seen that HP coated vesicles recorded a 17.5 ± 0.1% leak at the end of the first month (Fig. 7). Subsequently, the loss of drug was negligible indicating the possibility of across membrane osmotic gradiance to be operational which subsequently decreases, allowing a better packing of the membrane thus resulting in a better trapping efficiency of the system. The nature of the hydrophobized anchor did not reflect any appreciable change in the stability profile. On the other hand, plain niosomes showed 18.3 ± 1.5% leaching in the first 7 days, but in the next 21 days, niosomes degraded more rapidly leading to a 51.7 ± 1.5% leak after the first month. It was seen that vesicles lost their integrity and 89.1 ± 4.1% of drug leaching was recorded after 3 months of storage at ambient temperature. At the end of the long-term stability experiments, the majority of plain niosomes were found to be disrupted (Table 2). Similar trends were observed at lower (4 ± 1 °C) storage temperatures however, an overall slower leaching profile was recorded than at 37 ± 1°C (near phase transition temperature of the constituent lipids). The results are well anticipated and appreciated due to the higher stability of the vesicular membranes at lower temperatures (Fig. 8).

Niosomes coated with hydrophobized pullulan exhibited an exceptional stability profile against the challenges of pH, ionic strength, detergent and bile, osmotic pressure, simulated gastrointestinal milieu and long term storage. It could therefore be inferred that partially hydrophobized polysaccharide could effectively coat and impart biochemical and physicochemical stability to a niosomal bilayer. The stabilization of a niosomal membrane on coating with partially hydrophobized polysaccharide from the aqueous phase is very similar to nature’s way of stabilizing biological membranes.

The coated niosomal version, which has been found physico-chemically stable in the gastrointestinal milieu, may serve as an excellent antigen adjuvant system for oral immunization. Such systems may also be of great therapeutic potential where highly potent therapeutic moieties are to be administered, as this may offer carrier-content uptake by peyer’s patches. Work on coated niosomal peroral de-

![Fig. 6: Osmotic stress studies at different molar (hypotonic to hypertonic) to plasma concentrations of sodium chloride solutions. Plain niosomes •, OPPu coated niosomes ■, CHPu coated niosomes ▲.](image)

![Fig. 7: Long term stability studies at physiologic temperature (37 ± 1°C) estimated at different time intervals in terms of % drug latency.](image)

![Fig. 8: Long term stability studies at physiologic temperature (4 ± 1°C) estimated as the remaining drug per unit of surfactant per unit time (drug latency) at different time intervals.](image)

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### Table 2: Stability studies of various coated and uncoated niosomal systems

<table>
<thead>
<tr>
<th>Niosomal composition</th>
<th>Shelf stability in terms of % drug latency</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1 week</td>
</tr>
<tr>
<td></td>
<td>4 ± 1°C</td>
</tr>
<tr>
<td>Span 60:CH (7:3)</td>
<td>±</td>
</tr>
<tr>
<td>Span 60:CH:DCP (7:3:0.5)</td>
<td>±</td>
</tr>
<tr>
<td>(Span 60:CH:DCP):CHPu (7:3:0.5):0.1</td>
<td>±</td>
</tr>
<tr>
<td>(Span 60:CH:DCP):OPPU (7:3:0.5):0.1</td>
<td>±</td>
</tr>
</tbody>
</table>

All results are recorded in terms of % drug leaching values. * Leaching criteria are related to the amount of liposome bound Propranolol HCl at day zero or at the start of the incubation. (+) Leaching within accepted limits, 0-10%; (±) 10-25%; (-) 25-50%; (---) 50-75%; (---) >75%. (---) vehicles disrupted and released these contents.
livery for mucosal and humoral immunization is in process in our laboratory. The coated versions may equally be useful in bio-film or bacterial cell targeting where the uptake and subsequent carrier bio-processing is critical in negotiating drug release and pharmacodynamic effects. Therefore, the proposed carriers which could retain and sequester the content en route are considered to be ideal. However, the immunological consequences of the system are still to be explored and its targeting potential towards cell specific surface determinants remains to be investigated.

3. Experimental

3.1. Materials

Spain® 60, cholesterol and dietyl phosphate were purchased from Loba Chemie, India. Pallulan from Aurobasoldium pallulan was obtained from Sigma (St. Louis, MO, USA) and used without further purification. The model drug propranolol hydrochloride was a gift sample from MAC Laboratories Ltd., Mumbai, India. Other materials and reagents were of analytical grade (Qualigens, Chemical division of Glaxo India Ltd).

3.2. Methods

3.2.1. Synthesis of pallulan derivatives

Pallulan was derivatized with an O-palmitoyl anchor or a cholesterol moiety following the procedures of Hummelen and Wessel [17] or Sato [18]. O-Palmitoyl pallulan (OPP), in brief, was prepared by treating pallulan (1.0 g) in dry DMP at 60 °C to palmitoyl chloride (0.1 g) in DMP in the presence of dry pyridine (1.0 ml). The mixture was stirred at 60 °C for 6 h and slowly poured into absolute ethanol (100 ml) under vigorous stirring. The precipitate of OPP thus obtained was collected and washed with 50 ml of absolute ethanol and 5 ml of dry diethyl ether, and dried under vacuum at 50 °C for 1 h (Scheme 1). The cholesterol derivative of pallulan was synthesized as described [2] with appropriate modifications. In brief, carboxymethylated pallulan was obtained by the reaction of pallulan (1.0 g) with sodium monochloroacetate (0.95 g) in 1 M NaOH. The resulting solution was treated at pH 4.7 with ethylene diamine (1.25 g) in the presence of 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (0.5 g) as a coupling reagent. The amino-tether carboxymethylated pallulan thus obtained was reacted with cholesterol chloroformate (0.5 g) in a water free FMP solution at 60 °C for 24 h and the precipitate obtained was dried in vacuo at 50 °C (Scheme 2). The hydrophosphorylated derivatives of pallulan (HP) were subjected to FT-IR and 1H NMR spectrometry. The 1H NMR spectrum was obtained in deuterated DMSO (50 µl/ml) containing tetramethylsilane (TMS) as internal standard operating at a frequency of 425 MHz (Spectrometer Vario Unity-500). The IR spectrum of OPP and Pe (1%) incorporated in a KBr disc, was run on a FT-IR single beam spectrometer (Carl Zeiss, Specker 75, Germany).

3.2.2. Preparation of niosomes

Spain® 60, cholesterol and dietyl phosphate were taken in different mole fraction ratios (Table 1) and dissolved in a minimum quantity of diethyl ether in a rotary flash evaporator. The solvent was evaporated in order to coat the inside surface of the flask of the rotary flash evaporator (York, India). After vacuum desiccation of an air mixture for about 1 h, 500 µl of PBS (pH 7.4) containing 10 µg/ml of propranolol-HCl was added at 50 °C and the dispersion so obtained was allowed for complete hydration at an ambient (30 °C) temperature. The prepared vesicles were then dialyzed against PBS (pH 7.4) using dialysis tubes (Sigma, USA) for removal of any free drug. The dialyzed vesicles were centrifuged at 60,000 × g for 60 min and the pellets were re-suspended in PBS (pH 7.4) for polymer coating of the vesicle surface.

3.2.3. Polysaccharide coating of the vesicles

Capping of the niosomes with hydrophobized polysaccharides was accomplished by incubation of the vesicles and derivatized pallulan for a period of 6 h. Surface conductivity of the vesicles at different time intervals was recorded. Based upon these measurements, protein to surfactant ratio and incubation time were optimized (Systronics Conductivity Bridge, India). Hydrophobized pallulan dissolved in a minimum volume of mixed phosphate buffers (pH 7.4) were added to the previously prepared vesicular dispersion in 1:10 volume ratio total nonionic surfactant in 5.1 ± 10-4 g/ml mix. The dispersion mix was subsequently kept incubated in a microvascular for 6 h at ambient temperature followed by refrigeration temperature overnight. Uncoated niosomes without polymer were similarly treated. The coated niosomes were centrifuged (Beckman L8-55 Ultracentrifuge, 150,000 × g, 15 °C, 30 min) to remove unentrapped drug.

3.2.4. Separation of unbound material from the polysaccharide coated vesicles

Polyvinylcholride coated niosomes were isolated by gel chromatographic separation. A Sephadex G-50 column was equilibrated with mixed phosphate buffers and pre-saturated with niosome constituents (Length, 50 cm, diameter: 15 mm, flow rate: 150 µl/min, fraction volume: 2.0 ml/fraction). Fractions (2.0 ml chile) were collected and aliquots of 100 µl of the fractions were assayed for hydrophobized pallulan (HP) and niosomes (OD at 450 nm) using established procedures [23, 24].

3.3. Characterization of the coated niosomes

3.3.1. Vesicle size and distribution

Vesicular dispersions were appropriately diluted and wet mounted on a haemocytometer and photographed with a phase contrast microscope (Leitz, Bömmel, Germany). The negatives were projected on a piece of calibrated paper using a photographic enlarger (×1250). Diameter of approx. 500 vesicles were measured for each system and assigned to the nearest 1 µm size group.

3.3.2. Entrapment efficiency

Predialysed vesicular dispersions were centrifuged at 150,000 × g for 60 min as described [23]. Pellets obtained were re-suspended in 0.01 M PBS (pH 7.4) and the process was repeated 3 times. Vesicular dispersions were dialysed with 10% (w:v) propylene:PBS (1:1 v:v), and centrifuged again. The latterized contents was analyzed at 290 nm using a UV/VIS Spectrophotometer (Shimadzu DB 1601A, Japan) [25]. The entrapment efficiency was expressed as the ratio of experimentally measured amount of the drug in the dispersion and the added amount of the drug intended for encapsulation.

3.3.3. In vitro release profile

Pellets from unloaded and OP-PH and CH-PH coated niosomes (500 µl) were suspended in 2.5 ml of simulated gastric fluid (SGF) or simulated intestinal fluid (SIF), and placed in a Sigma dialysis bag at 37 ± 1 °C. The bags were incubated at 37 ± 2 °C in a metabolic shaker (York, India). Samples of 25 µl were removed at various time intervals up to 24 h, centrifuged (10,000 rpm, 25 °C, 10 min) and the supernatants were analyzed at 289 nm for the released (leached) drug [25].

3.3.4. Detergent challenge and freeze thawing studies

Structural integrity of niosomal bilayers coated with hydrophobized pallulan was measured against the challenge of detergent (deoxy-PEG) and freeze thawing. Drug loaded plain niosomes were pre-incubated with gradually increasing concentrations of hydrophobized pallulan for 60 min. Drug leakage was continuously estimated and deoxy-PEG (0.07% w/v) was added. The rate of drug release from different hydrophobized pallulans was plotted as a percentage of drug release per minute. The maximum release rate was measured following the addition of 0.1% w/v Triton X-100. For freeze-thawing studies, the vesicular dispersions were dialyzed with 0.05 M PBS (pH 7.4) to a final concentration of 0.5 µg surfactant/ml of suspension using increasing concentrations of HP Samples were rapidly frozen in liquid nitrogen and diluted slowly at room temperature. The drug release was measured immediately as described earlier [25].

3.3.5. In vivo stability with bile salts and fresh bile

Stability of vesicle and encapsulated propranolol against simulated bile salt solutions and against fresh rat bile was appraised by measuring the amount of intact (unchanged) drug associated with vesicles after 6 h incubation. The experiment was conducted with cholate, deoxycholate, taurocholate, glycocholate and glycodeoxycholate dissolved in phosphate buffered saline (pH 7.4) to a final concentration of 200 µg/ml. The stability of coated niosomes, in various bile salt solutions at concentrations below, at and above critical micelle concentrations (CMC) was recorded at 37 ± 1 °C by monitoring % of initial content. The same experimental conditions were used for incubation studies with freshly pooled albino rat bile already separated using established procedures. At different incubation times, aliquots of vesicular dispersions were removed, centrifuged and supernatants were analyzed for the drug content. From the data obtained % of initial content were calculated.

3.3.6. Osmotic stress studies

Effect of osmotic challenge on vesicle size and structural integrity was investigated by monitoring the variations in vesicle size, i.e., reduction or expansion of vesicular mean diameter (Phase contrast microscope, Leitz, Bömmel, Germany) upon incubation with different molar solutions of sodium chloride. The stress study consisted of incubation of small vesicles (100-500 µl) of different tonicity solutions to the vesicular dispersion (1.0 ml) in glass ampoules flushed with nitrogen. Aliquots of samples withdrawn over a period of 24 h were allowed appropriately and estimated for mean vesicle size.
5.3.7. Long term stability studies

Vesicular dispersions of various compositions (2.0 ml) were kept in amber colored glass ampoules flushed with nitrogen and stored at 37 ± 1°C and 4 ± 1°C for a period of 6 months. At different incubation times, aliquots of the vesicular dispersions were removed, centrifuged at 60,000 g for 60 min and supernatant was analysed for leached drug at 290 nm. The maximum level of drug leaching was measured after the addition of 0.1% w/v Triton X-100. The remaining drug per unit of surfactant per unit time was estimated at different time intervals (drug latency). Stability of the vesicles was checked (during storage) by their visual appearance with respect to aggregation and mean vesicle size, vesicles remaining per mm³, and by measuring the leached drug as a function of time at experimental temperatures.

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Ligand–Receptor-Mediated Drug Delivery: An Emerging Paradigm in Cellular Drug Targeting

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ABSTRACT: Receptor-mediated cellular events have received major attention in the field of drug/gene delivery in the past few years. These events, which are mediated through the endogenous ligands/epitopes, could be exploited for designing site-specific and target-oriented delivery systems. The past decade has seen the development of endogenous ligands and their mimics of exogenous origins to selectively deliver the contained or immobilized moieties to the cellular interiors using a wide range of cell surface receptors/epitopes. Ligand-mediated active targeting has emerged as a novel paradigm in targeting either vascular compartment (first-order), cellular (second-order), or intracellular (third-order) levels. Most carrier systems or bioconjugates explored so far can be used as cargo units for the site-specific presentation and delivery of various bioactives using biorelevant ligands, including antibodies, polypeptides, oligosaccharides (carbohydrates), viral proteins, fusogenic residues, and molecules of endogenous origin. In this review, we describe various ligand-receptor systems that have been investigated to date for targeted or cellular drug delivery. These include blood carbohydrate (lectin) receptors, Fc receptors, complement receptors, interleukin receptors, lipoprotein receptors, transferrin receptors, scavenger receptors, receptors/epitopes expressed on tumor cells, and cell adhesion receptors. The role of receptors as molecular target has opened new opportunities for cellular or intracellular targeting using carrier systems appended with targeting handles (ligands). Research in the field of ligand–receptor-based targeted system is expected to be an armamentarium and the focal point of research in the next millennium.

KEYWORDS: ligand, receptor, receptor-mediated endocytosis, ligand-mediated targeting, drug delivery
Endogenous carriers and ligands in non-immunogenic site-specific drug delivery

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Abstract

Targeted drug delivery has gained recognition in modern therapeutics and attempts are being made to explore the potentials and possibilities of cell biology related bioevents in the development of specific, programmed and target oriented systems. The components which have been recognized to be tools include receptors and ligands, where the receptors act as molecular targets or portals, and ligands, with receptor specificity and selectivity, are trafficked en route to the target site. Although ligands of exogenous or synthetic origin contribute to the selectivity component of carrier constructs, they may impose immunological manifestations of different magnitudes. The latter may entail a continual quest for bio-compatible, non-immunogenic and target orientated delivery. Endogenous serum, cellular and extracellular bio-ligands interact with the colloidal carrier constructs and influence their bio-fate. However, these endogenous bio-ligands can themselves serve as targeting modules either in their native form or engineered as carrier cargo. Bio-regulatory, nutrient and immune ligands are sensitive, specific and effective site directing handles which add to targeted drug delivery. The present review provides an exhaustive account of the identified bio-ligands, which are not only non-immunogenic in nature but also site-specific. The cell-related bioevents which are instrumental in negotiating the uptake of bio-ligands are discussed. Further, a brief account of ligand–receptor interactions and the set of biological events which ensures ligand-driven trafficking of the ligand–receptor complex to the cellular interior is also presented. Since ligand–receptor interaction is a critical pre-requisite for negotiating cellular uptake of endogenous ligands and anchored carrier cargo, an attempt has been made to identify differential expression of receptors and bio-ligands under normal and etiological conditions. Studies which judiciously utilized bio-ligands or their analogs in negotiating site-specific drug delivery have been reviewed and presented. Targeted delivery of bioactives using endogenous bio-ligands offers enormous options and opportunities through carrier construct engineering and could become a future reality in clinical practice. © 2000 Elsevier Science B.V. All rights reserved.

Keywords: Endogenous; Bio-ligand; Serum component; Receptor; Site specificity; Targeting

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Ligand directed macrophage targeting of amphotericin B loaded liposomes

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Abstract

Two types of ligand anchored multilamellar liposomes (MLVs) containing amphotericin B (Amp B) were prepared. The MLVs consisting of soya phosphatidylcholine (PC) and cholesterol (Chol) were coated with O-palmitoyl mannan (OPM). Similarly, the MLVs with the same Amp B content consisting of soya PC, Chol and phosphatidylethanolamine (PE) were prepared and covalently anchored with p-aminophenyl-mannopyranoside (PAM). The surface modified MLVs and their plain counterparts were characterised for size, shape, lamellarity, entrapment efficiency and ligand density. The stability in serum and in vivo bio-distribution in albino rats were also determined. It was observed that extent of accumulation of liposomal Amp B in macrophage rich organs, particularly liver, spleen and lungs was significantly high when compared against the free drug. The rates and extent of accumulation were found to increase further on ligand anchoring. In either of the cases, the macrophagic uptake of ligand anchored liposomes was inhibited significantly on pre-injection of hydrolysed mannan, being suggestive of receptor mediated uptake of ligand anchored liposomes. Comparison of biodistribution pattern of ligand anchored MLVs revealed that PAM linked liposomes exhibited a higher hepatosplenic accumulation where as drug accumulation in lungs was highest in the case of OPM coated liposomes. It was thus observed that mannopyranoside is a specific ligand for targeting bioactives to the macrophages of liver and spleen while OPM could preferentially negotiate the targeting of bioactives to the alveolar macrophages. © 2000 Elsevier Science B.V. All rights reserved.

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

1. Introduction

Targeted drug delivery to the macrophages has been appreciated as a strategy for achieving diverse objectives like treatment of lysosomal storage diseases (Gordon and Rabinowitz, 1989), targeting of immunomodulators to activate macrophages (Fidler, 1988), cell or cell product depletion (van Rooijen and Sanders, 1994) and blockade of the macrophages (O’Mullane et al., 1987). Many approaches for targeting the drugs to the macrophages have been developed, which are largely represented by liposomes (Alving, 0378-5173/00 $ - see front matter © 2000 Elsevier Science B.V. All rights reserved.
PH: S0378-5173(00)00522-6
1982; Bakker-Woudenberg et al., 1994). Although liposomes show natural affinity towards the macrophages and passively targeted to them; yet inclusion of the macrophage receptor(s) specific ligands may significantly enhance the rates and extent of liposomal uptake by the macrophages.

Amphotericin B (Amp B) is a drug of choice in systemic fungal infections (Walsh and Pizzo, 1990). The drug is also used in the treatment and management of visceral and mucocutaneous leishmaniasis, and in pulmonary aspergillosis (Sarosi, 1990; Denning and Stevens, 1990). Amp B manifests serious adverse complications related to dose dependent acute and chronic toxicity. For diseases of microbial etiology, the intracellular localisation of the pathogens necessitates the administration of relatively high doses of the cytotoxic drugs for the effective killing of the pathogens, thereby causing the side effects. The rationale approach to the problem requires that drugs should be targeted to the macrophages in such a way that the interaction of the free drug with non-target tissues could be minimised. Maximum tolerated dose of Amp B is considerably low in mice; LD_{50} is 1.2 mg kg^{-1} and doses higher than 1.6 mg kg^{-1} cause acute toxic reactions followed by cardiac respiratory arrest (Lopez-Berestein et al., 1983). Treatment of disseminated fungal infections by liposomal Amp B results in a lower toxicity and significantly increased survival times (Brautigum et al., 1990). It has been proposed that increased concentrations of drug in macrophages through passive liposomal uptake may improve its therapeutic index (Janknegt et al., 1992; de Marie et al., 1994). Toxicological comparison of free and liposomally formulated Amp B in mice has revealed that maximum tolerated dose has been significantly increased. The LD_{50} has been found to increase from 1.2 to more than 12 mg kg^{-1} in the case of mice (Lopez-Berestein et al., 1983). Although liposomal formulations of Amp B could considerably reduce the toxicity of the drug and subsequently make it possible to enhance the therapeutic index, however, their pharmacokinetic and pharmacodynamic profile still needs to be improved.

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

It was expected that ligand mediated active targeting to the macrophages would significantly increase the rate and extent of macrophage accumulation of drug. This may reduce the required doses of liposomal Amp B in diseases like hepatosplenic fungal infections and leishmaniasis and in pulmonary aspergillosis associated with granulocytopenia. Mannose/fucose receptors, expressed abundantly in liver, spleen and alveolar macrophages have been most widely utilised for targeting bioactives to the macrophages (reviewed in Vyas, 2000). The receptor facilitates endocytosis of glycoproteins terminated with mannose, fucose and glucosamine. Mannose residues have been appended to liposomes as mannosylated albumin (Garcon et al., 1988); palmitoylated polysaccharides (Sunamoto et al., 1985); mannosio- arachidonic esters (Yachi et al., 1995); mannos terminated glycolipids (Barrat et al., 1986); mannopyranoside (Bachhawat et al., 1984) and mannose terminated glycoproteins (Szoka and Mayhew, 1983; Ponpipon et al., 1984) for macrophage targeting.
The present work was programmed for designing an actively targeted system of Amp B based on liposomes. The macrophages being the target were assessed for selective accessibility through receptor-mediated endocytosis using O-palmitoylated mannan (OPM) and p-aminophenylmannopyranoside (PAM) as specific ligand modules. Comparative in vivo distributions and targeting profiles of OPM and PAM anchored liposomes against plain liposomes were studied.

2. Materials and methods

2.1. Materials

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

2.2. Preparation of OPM coated MLVs containing Amp B

2.2.1. Preparation and optimisation of MLVs containing amphotericin B

Multilamellar vesicles (MLVs) containing Amp B were prepared by the method described by Lopez-Berestein et al. (1983). Soya PC and Chol were dissolved in the minimum amount of chloroform and a methanolic solution (60 μg ml⁻¹) of Amp B was added to it. Soya PC to Chol ratio (8:2 molar ratio) was kept constant while Amp B content was varied at different molar percent ratio levels, i.e. (20, 16, 12, 8, 4, 2 and 1% moles of the total lipids) in different preparations for determining optimum Amp B content. The organic solvent mixture was removed using a rotary flash evaporator under reduced pressure. The dried film was hydrated with 0.9% NaCl solution at ±40°C for 60 min and subsequently at room temperature for 6 h. The dispersion was centrifuged at 60 000 rpm for 4 h and the pellet was resuspended in 0.9% NaCl solution.

The liposomal formulations were centrifuged through Sephadex G-50 mini-column at 2000 rpm for 3 min for the separation of unentrapped drug. The liposomal fraction was added with minimum amount of triton X-100 (0.5% w/v), drug content was determined spectrophotometrically at 404 nm and percent drug entrapment was calculated. MLVs with optimum Amp B to lipid ratio were optimised for optimum Soya PC to Chol ratio in terms of percent drug entrapment and toxicity towards erythrocytes. The MLVs with different Soya PC to Chol ratios (90:10, 80:20, 70:30, 60:40, 50:50 molar ratios) were prepared. Amp B content however was kept constant at its optimum concentration level. The liposomes were evaluated for percent entrapment and toxicity to mammalian cells in terms of percent hemolysis. Percent hemolysis was determined by the method described by Mehta et al. (1984). Liposomal dispersions containing equivalent amount of Amp B (50 μg ml⁻¹) were incubated with 1.0 ml mammalian blood at 37 ± 1°C for 45 min, centrifuged at 60 000 rpm for 4 h and haemoglobin released in the supernatant was measured spectrophotometrically at 550 nm (Mehta et al., 1984). For control, blood was similarly incubated with 0.9% NaCl solution. Blood was incubated with same volume of distilled water (100% hemolysis). From the haemoglobin released in supernatant, percent hemolysis in each case was computed and assessed. Percent hemolysis and percent entrapment were plotted against Soya PC to Chol ratio, from which optimum Soya PC to Chol ratio was determined.

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

O-palmitoylated mannan was synthesised from the yeast extract by the process reported elsewhere in the literature (Hammerling and Westphal, 1967). O-palmitoyl mannan, in brief, was prepared by reacting mannan (1.0 g) in dry DMF at 60°C with palmitoyl chloride (0.1 g) in DMF in the presence of dry pyridine (1.0 ml). The mixture was stirred at 60°C for 6 h and slowly poured into
absolute ethanol (100 ml) under vigorous stirring. The precipitate of OPM, thus obtained was collected and washed with 50 ml of absolute ethanol and 25.0 ml of dry diethyl ether, and dried in vacuo at 50 ± 1°C for 1 h.

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

Coating of the Amp B loaded MLVs with hydrophobised derivative of mannan was affected by incubating 1.0 ml of plain liposomal suspension with the dispersion of optimised amount of OPM (0.15:1 w/w OPM:PC) in 0.9% w/v NaCl solution. The dispersion was stirred gently at room temperature for 4 h. Excessive, unbound polysaccharide was removed by spinning the resulting suspension through a sephadex G-50 mini-column at 2000 rpm for 5 min. For optimisation of OPM:PC ratio and incubation time required for the effective coating, positively charged MLVs were prepared by incorporating stearylamine as one of the phospholipids at 0.5 mole% level of the total lipidic contents. These MLVs were incubated with varying amounts of OPM, i.e. w/w ratios based on PC weight (0.01:1, 0.05:1, 0.1:1, 0.15:1, 0.2:1, 0.25:1 and 0.5:1 w/w ratio) for 4 h. After removing excessive, unbound polysaccharide specific electrical conductance of these liposomes was determined (Jaitely and Vyas, 1999). Conductivity was measured at an applied voltage of 10 mV using Systronics Conductivity Bridge 305 (India) and the measurements were made in micromhos. Specific electrical conductance was plotted against the OPM:PC ratio and optimum OPM:PC ratio was determined from the plot as one, at or beyond which no further changes in the conductance recorded. Similarly, to obtain optimum incubation time, formulations were incubated at optimum OPM concentration for different time intervals (0.5, 1.0, 1.5, 2.0, 2.5, 3.0 and 4.0 h), and specific electrical conductance of these vesicles was recorded after separating the excessive, unbound polysaccharide. From the plot of Specific electric conductance against incubation time, optimum incubation time was determined to be one, at or beyond which no significant changes in the conductance were recorded.

2.3. Preparation of mannopyranoside (PAM) linked MLVs containing Amp B

2.3.1. Preparation of PE based liposomes

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

2.3.2. Linkage of mannopyranoside (PAM) to MLVs

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

2.4. Characterisation of liposomes coated with mannose terminating ligands

Developed formulations were characterised prior to and after surface ligand anchoring. The different liposomal formulations were evaluated for their shape and vesicle type by transmission electron microscopy (TEM). Vesicle characterisation for size and shape was performed using TEM. Phosphotungstic acid (1%) was used as a negative stain (JEM 1200, EX 11, JEOL, Japan). Carbon coated samples were placed over a copper grid and subjected to TEM analysis. Vesicle size distribution was also assessed using a phase contrast...
microscope (Leitz, Biomed, Germany). Vesicle dispersions were appropriately diluted and wet mounted on a haemocytometer and photographed using the microscope. The negatives were projected on a piece of calibrated paper using an enlarger with an adjusted magnification (×1250). Diameters of around 500 vesicles were noted for each system and average vesicle size was calculated.

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

2.5. Stability in serum

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

2.6. Determination of in vivo target specificity

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

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

\[
\text{Drug localization index} = \frac{\text{Drug concentration in target tissue at time } 't' \text{ after administration of test delivery system}}{\text{Drug concentration in target tissue at time } 't' \text{ after administration of free drug}}
\]
3. Results and discussions

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

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

At higher concentrations (8–20 mole% of the total lipids) of Amp B ribbons and unspecified structures were observed. However, as the concentration of Amp B was gradually lowered, relative numbers of ribbons and unspecified structures decreased while number of liposomes increased (Table 1). It is speculated that the associations of Amp B molecule with Chol may result in leaky membrane formations leading to lower entrapped volume and hence lower size of the vesicles. This hypothesis however, needs confirmation from experimental studies. When molar concentration of Amp B was used at 2% of the total lipids, liposomal formulation was be free of other undesired structures and average vesicle size measured was 2.37 ± 0.76 μm.

In another variation, with an increase in Chol concentration in liposomes at constant and optimised concentration of Amp B, distinctive changes in toxicity to the erythrocytes and percent entrapment of Amp B were recorded. On increasing the molar ratio of Chol:PC from 10:90 to 40:60 the percent hemolysis was recorded between 11.2 ± 1.09 and 1.0 ± 0.19% (Fig. 1). The mechanism responsible for toxicity reduction of Amp B upon liposomal entrapment is still undefined. However, it seems to be attributed to the more stable and compact configuration of bilayers and intercalation of Amp B in the bilayers. Probably increased interaction of Amp B molecules with cellular Chol restricts their lateral partitioning with Chol present in the erythrocyte membranes. An increase in the concentration of Chol however resulted in a relatively low percent entrapment of Amp B (Fig. 1). It may probably be due to stericly favourable and hence, preferential accommodation of the Chol molecules with in the bilayer assemblages. Optimum PC:Chol molar ratio was found to be 7:3, which could entrap maximum amount of drug (79.2 ± 1.29) with acceptably lower levels of toxicity towards erythrocytes (percent hemolysis = 1.1 ± 0.18).

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

<table>
<thead>
<tr>
<th>Amp B contenta</th>
<th>Types of structures formed</th>
<th>Entrapment efficiency (n = 3)</th>
</tr>
</thead>
<tbody>
<tr>
<td>20</td>
<td>Comma shaped ribbons and other unspecified structures</td>
<td>Not determined</td>
</tr>
<tr>
<td>16</td>
<td>Unspecified structures, ribs and some liposomal population</td>
<td>Not determined</td>
</tr>
<tr>
<td>12</td>
<td>Distorted liposomes, ribbons and some intact liposomes</td>
<td>Not determined</td>
</tr>
<tr>
<td>8</td>
<td>Mostly liposomes, some ribbons and distorted liposomes</td>
<td>Not determined</td>
</tr>
<tr>
<td>4</td>
<td>Only liposomes of size range lower than 1.6–4.8 μm with average size 1.86 μm</td>
<td>68.9 ± 1.23%</td>
</tr>
<tr>
<td>2</td>
<td>Only liposomes of size range lower than 1.6–6.4 μm with average size 2.37 μm</td>
<td>79.2 ± 1.56%</td>
</tr>
<tr>
<td>1</td>
<td>Liposomes of size range 1.6–4.8 μm with average size 3.86 μm</td>
<td>84.1 ± 1.33%</td>
</tr>
</tbody>
</table>

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

The OPM:PC ratio and incubation time were optimised by measuring the changes in electrical conductance of stearylamine containing liposomal suspension with varying concentrations of OPM as well as with variation in incubation time. A decrease in electrical conductance was observed from 3306.4 ± 12.1 to 2348.2 ± 11.2 μmhos, when the OPM:PC ratio was increased from 0.01:1 to 0.15:1 (Fig. 2a). It apparently relates to the extent of masking of surfacial charge (contributed by stearylamine) by OPM. Electrical conductance, however, remained nearly constant on further addition of OPM. This indicates no further charge based interaction of the components of the bilayer and is suggestive of the completion of coating. The optimum OPM:PC ratio was found to be 0.15:1, while optimum incubation time (the time that corresponds to completion of OPM coating under the experimental condition) recorded was 1.5 h (Fig. 2b). At the optimum OPM concentration level, the optimum incubation time was recorded to be 1 h after which electrical conductance remained fairly constant at 2388 ± 12.7 μmhos.

The other liposomal formulation was prepared from PC and PE as constitutive lipids and optimised for parameters including PC to PE ratio and Chol to total lipid ratio. The optimisation was carried out to get an optimum vesicle size coupled with a higher entrapment and an acceptable toxicity towards erythrocytes. PE containing liposomes were evaluated and optimised for total PE content. It was found that additional PE compensated with an equivalent reduction in PC exhibited no significant effect on the toxicity of Amp B towards erythrocytes as well as on percent entrapment so far the concentration of Chol was kept
constant (Table 2). However, when PE:PC ratio raised near 1:1 molar ratio, the vesicles were off spherical in shape and distorted vesicles were seen. This can be attributed to the reported instability of the PE because of its rapid phase transition to non-bilayer (hexagonal phase II) structures (Litzinger and Huang, 1992). However, when the PE:PC ratio was decreased relatively stable vesicles were obtained, owing to increased stabilising contribution of PC and Chol and hence less membrane defects. Optimum PC:PE:Chol ratio was found to be 5:2:3 mM. Liposomes formed at this composition were absolutely spherical within a size range 1.66 to 4.98 μm. 87 ± 2.5% of liposomal population was lower than 3.50 μm in size. The average size of the vesicles was 1.78 ± 0.35 μm, which increased marginally on anchoring PAM residues via glutaraldehyde spacer arm (Table 3).

The optimised formulations used for further in vitro and in vivo studies were coded as PC3 (PC liposomes-plain), OMPPC3 (OPM coated), CE3 (PC/PE liposomes-plain), and PAMCE3 (PAM coated CE3). Ligand appended liposomes were characterised for vesicle shape, size and size distribu-

Photomicrographs show the surface coating/anchoring of ligands on the amphotericin B loaded liposomes.
1. Coating is signified with extinguished multilamellarity and opaque appearance.
2. Surface anchoring and inter-digitization of mannose-terminating ligand (PAM).
3. Coating of hydrophobized derivative of mannan (OPM).
Table 2
Optimisation of PC:PE:Chol ratio

<table>
<thead>
<tr>
<th>Molar ratios PC:PE:Chol</th>
<th>Entrapment (%)&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Hemoysis (%)&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>7.0:0.0:3.0</td>
<td>79.2 ± 1.12</td>
<td>1.5 ± 0.021</td>
</tr>
<tr>
<td>6.0:3.0:1.0</td>
<td>81.6 ± 1.24</td>
<td>13.1 ± 0.85</td>
</tr>
<tr>
<td>6.0:2.0:2.0</td>
<td>78.9 ± 1.18</td>
<td>6.9 ± 0.54</td>
</tr>
<tr>
<td>5.0:2.0:3.0</td>
<td>74.2 ± 1.21</td>
<td>1.6 ± 0.036</td>
</tr>
<tr>
<td>4.0:3.0:3.0</td>
<td>70.0 ± 1.24</td>
<td>1.8 ± 0.054</td>
</tr>
<tr>
<td>4.0:4.0:2.0</td>
<td>69.8 ± 1.54</td>
<td>7.3 ± 0.63</td>
</tr>
</tbody>
</table>

<sup>a</sup> All the values are representatives of mean ± S.D. for three independent determinations.

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

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

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

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

<table>
<thead>
<tr>
<th>Formulation codes</th>
<th>Composition (mmole)</th>
<th>Nature of ligand</th>
<th>Percent encapsulation&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Average vesicle size&lt;sup&gt;c&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>PC3</td>
<td>PC:Chol (7:3)</td>
<td>-</td>
<td>79.6 ± 1.8</td>
<td>2.35 ± 0.25</td>
</tr>
<tr>
<td>OPMPC3</td>
<td>PC:Chol (7:3)</td>
<td>OPM</td>
<td>78.6 ± 2.1</td>
<td>3.04 ± 0.18</td>
</tr>
<tr>
<td>CE3</td>
<td>PC:PE:Chol (5:2:3)</td>
<td>-</td>
<td>74.2 ± 1.7</td>
<td>1.78 ± 0.35</td>
</tr>
<tr>
<td>PAMCE3</td>
<td>PC:PE:Chol (5:2:3)</td>
<td>PAM</td>
<td>73.9 ± 1.0</td>
<td>2.10 ± 0.06</td>
</tr>
</tbody>
</table>

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

The biodistribution patterns studied clearly indicate the superiority of the liposomal Amp B as compared against the plain drug in increasing the accumulation of Amp B in the organs rich in macrophages (liver and spleen). The intravenous administration of free Amp B (0.8 mg kg$^{-1}$ body weight) resulted in relatively lower plasma concentrations of Amp B (0.9 ± 0.12 μg ml$^{-1}$) after 15 min which further declined to 0.2 ± 0.1 μg ml$^{-1}$ as estimated at 4 h and to negligible extent (not detectable) at 24 h. This could account for the rapid but non-specific tissue distribution of the free drug to various tissues of the body. This seems related to relatively low levels of drug recovered from macrophage rich organs like liver, spleen and lungs as compared with Amp B bearing liposomal formulations. Plasma concentrations of Amp B after the administrations of Amp B bearing plain liposomes were significantly higher than the free drug, i.e. 4.6 ± 0.7 and 4.9 ± 1.1 μg ml$^{-1}$ after 15 min, respectively, in the case of formulations PC3 and CE3 (Fig. 4). Ligand anchored liposomes (OPMPC3 and PAMC3E3) exhibited still higher plasma concentrations (3.1 ± 0.9 and 2.9 ± 1.1 μg ml$^{-1}$, respectively, after 15 min) than free drug, but it was noticeably lower than that obtained after the administration of their plain counterparts (PC3 and CE3).

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

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

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

The hepato-splenic quantitative uptake of OPM coated and PAM linked liposomes were lowered when 10.0 mg hydrolysed mannan was administered intravenously prior to injecting the formulations (Fig. 5A and B). This qualitatively
suggests the possible involvement of mannose receptors expressed onto the membrane of macrophages. The later may be involved in the selective and higher uptake of the ligand appended liposomes. The faster rates and higher extent of liposome uptake by liver, spleen and lungs on anchoring mannose terminated ligands to them has been reported by various workers (Ghosh and Bacchawat, 1980; Szoka and Mayhew, 1983; Das et al., 1985). However, the increased macrophage uptake is not completely inhibited by the pre-injection of hydrolysed mann, which suggests that some other mechanisms besides mannose/fucose receptor mediated endocytosis are also involved in promoting and contributing to an enhanced macrophage uptake. We speculate that probably uptake via anionic scavenger receptor mediated endocytosis is in part responsible for the observed higher uptake. This hypothesis, however, need confirmation through the competitive inhibition studies.

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

<table>
<thead>
<tr>
<th>Codes</th>
<th>Organ</th>
<th>Percent dose recovered after</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>15 min</td>
</tr>
<tr>
<td><strong>Free Amp B</strong></td>
<td>Liver</td>
<td>21.6 ± 1.3</td>
</tr>
<tr>
<td></td>
<td>Spleen</td>
<td>4.6 ± 0.01</td>
</tr>
<tr>
<td></td>
<td>Lung</td>
<td>2.3 ± 0.01</td>
</tr>
<tr>
<td></td>
<td>Kidney</td>
<td>6.8 ± 0.01</td>
</tr>
<tr>
<td><strong>PC3</strong></td>
<td>Liver</td>
<td>36.9 ± 0.1</td>
</tr>
<tr>
<td></td>
<td>Spleen</td>
<td>8.1 ± 0.01</td>
</tr>
<tr>
<td></td>
<td>Lung</td>
<td>3.1 ± 0.01</td>
</tr>
<tr>
<td></td>
<td>Kidney</td>
<td>ND^b</td>
</tr>
<tr>
<td><strong>OPM coated PC3</strong></td>
<td>Liver</td>
<td>42.1 ± 1.6</td>
</tr>
<tr>
<td></td>
<td>Spleen</td>
<td>12.4 ± 0.7</td>
</tr>
<tr>
<td></td>
<td>Lung</td>
<td>7.9 ± 0.3</td>
</tr>
<tr>
<td></td>
<td>Kidney</td>
<td>ND^b</td>
</tr>
<tr>
<td><strong>CE3</strong></td>
<td>Liver</td>
<td>31.8 ± 1.1</td>
</tr>
<tr>
<td></td>
<td>Spleen</td>
<td>6.3 ± 0.8</td>
</tr>
<tr>
<td></td>
<td>Lung</td>
<td>2.1 ± 0.2</td>
</tr>
<tr>
<td></td>
<td>Kidney</td>
<td>ND^b</td>
</tr>
<tr>
<td><strong>Mannopyranoside linked CE3</strong></td>
<td>Liver</td>
<td>47.9 ± 2.5</td>
</tr>
<tr>
<td></td>
<td>Spleen</td>
<td>14.4 ± 1.5</td>
</tr>
<tr>
<td></td>
<td>Lung</td>
<td>5.1 ± 0.2</td>
</tr>
<tr>
<td></td>
<td>Kidney</td>
<td>ND^b</td>
</tr>
</tbody>
</table>

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

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

4. Conclusion

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

Acknowledgements

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

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

References


