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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. Hydrophobized polysaccharides, O-palmitoyl pullulan (OPPu) and cholesteroyl pullulan (CHPu) 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 hydrophobized 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 ° and at 4 ± 1 °C establishes the potential of polysaccharide coated niosomes as an oral delivery system for water-soluble agents. Results from OPPu and CHPu 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 osmotic-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 electrophoretic mobility and makes it positive with the inclusion of stearylamine or negative with dicetyl phosphate, thus preventing niosomal fusion/swelling or aggregation [10]. In addition, the entrapment of hydrophobic drugs [11] or macromolecular prodrug [5] also increases the stability of these dispersions. Decreasing the air-water interface prevents the crystallization of these self-assembled surfactant monomers [12]. Further, it may be possible to stabilize niosomes by a variety of methods such as the addition of polymerized surfactants to the formulation, the use of membrane spinning lipids and the interfacial polymerization of surfactant monomers in situ [9].

The work describes the oral delivery potential of a polysaccharide appended niosomal system. Polysaccharide has been immobilized on the outer half of the bilayer with the help of hydrophobic anchors adapting palmitoylation and cholesteroyl esterification. Capped niosomes were challenged against harsh environments and physicochemical stimuli to mimic the biological stresses to be encountered in the biofluids. The role of partially hydrophobized anchors on the stability and integrity of the coated niosomes has also been discussed.

2. Investigations, results and discussions

Sunamoto et al. [13–15] succeeded in assembling an artificial cell wall on the outermost surface of liposomes by partially hydrophobized polysaccharides and reported the effective trapping of a water-soluble fluorescent marker probe. These vesicles demonstrated exceptional permeability characteristics and biochemical stability against the challenges of plasma, serum and elevated temperatures. In addition, the enzymatic lysis of the liposomal membrane by phospholipase D was arrested and addressed as ascertained by the fluorescence quenching based experiments. These results encouraged us to employ a polysaccharide net over the niosomal membrane to create an improved and stable vehicle, in particular for the oral delivery of drugs/macromolecules.

2.1. Characterization of hydrophobized polysaccharides (HP)

Pullulan is a linear α-glucan, produced by the yeast like fungus *Pullunaria pullulan*, in which about 480 maltotriose units are linked by $\alpha(1 \rightarrow 6)$ glycosidic bonds. Like 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 vesicular 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 cholesteroyl 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 cholesteroyl esterification (CHPu) (Schemes 1 and 2). Pullulan derivatives get inserted/interdigitized into the bilayer on incubation via hydrophobic anchors, extending the polysaccharide portion into the aqueous milieu. The derivatization products were characterized by IR- and ^{1}H 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 ^{1}H 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 cholesteroyl 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.

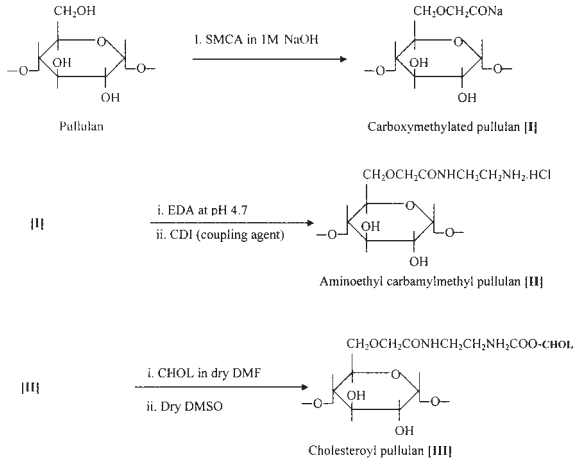
2.2. Characterization of polysaccharide coated niosomes

An artificial cell wall consisting of hydrophobic palmitoyl and cholesteroyl 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 hydrophilic 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 conductance of the vesicles stabilized at a lower ratio of CHPu to surfactant (0.01 : 1) than of OPP to surfactant (0.1 : 1). This could be due to the fact that CHPu could have filled the locus 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 vesicly 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 bifringes revealed that coated vesicles were relatively larger in mean size than plain niosomes (uncoated). This may be accounted to the polymeric capping, a dual diffusion barrier on the niosomal surface.

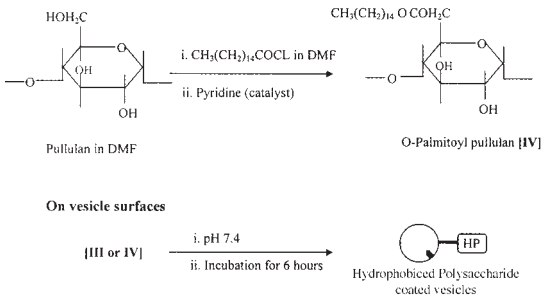
Encapsulation efficiency of drug in CHPu and OPPu compositions were 27.5 ± 0.75 and 30.1 ± 0.7, respectively,

Scheme 1



Abbreviations
SMCA = Sodium monochloroacetate, EDA = Ethylene diamine, CDI = 1-Ethyl-3-(3-dimethylamino)-propylcarbodiimide, CHOL = Cholesterol, DMF = Dimethyl formamide, DMSO = Dimethyl sulfoxide

Scheme 2



Scheme 3

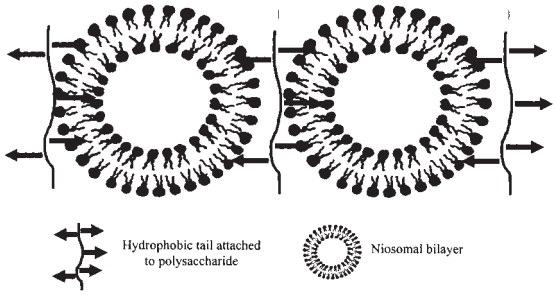


Table 1: Composition and characterization of various niosomal systems

Composition	Molar ratio	Shape (phase contrast microscope)	Average vesicle size	Encapsulation efficiency
Span 60 : CH	(7 : 3)	Multilamellar, bifringes visible	2.7 ± 1.2	32.7 ± 1.2
Span 60 : CH : DCP	(7 : 3 : 0.5)	Multilamellar, bifringes visible	2.9 ± 1.2	35.5 ± 0.8
(Span 60 : CH : DCP) : CHPu	(7 : 3 : 0.5) : 0.1	Lamellarity indistinguishable; opaque appearance	3.7 ± 0.9	27.8 ± 0.6
(Span 60 : CH : DCP) : OPPu	(7 : 3 : 0.5) : 0.1	Lamellarity indistinguishable; opaque appearance	3.5 ± 0.75	30.4 ± 0.6

revealing that CHPu and OPPu 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 pre-

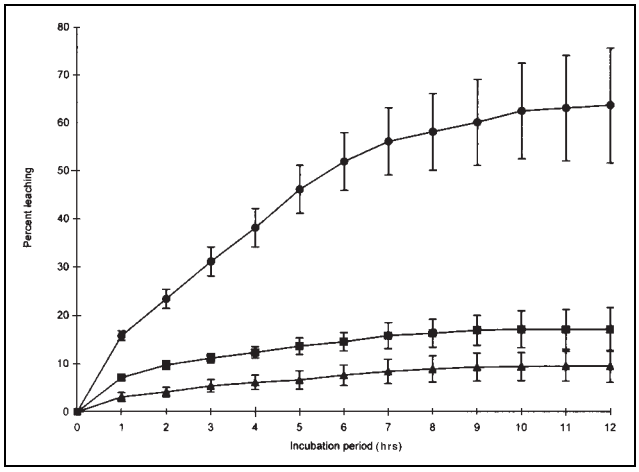


Fig. 1: Drug leaching studies in the simulated gastric fluid (pH 1.2). Plain niosomes ●; OPPu coated niosomes ■; CHPu coated niosomes ▲

sents % drug leached as a function of time recorded over various time intervals (1, 6, and 12 h). It could be seen that CHPu coated niosomes were able to retain $90.5 \pm 0.7\%$ of the drug, while OPPu 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 OPPu coated niosomes than in CHPu coated niosomes. The results recorded in terms of drug leaching are significantly better than that of the plain niosomal system which could retain $32.5 \pm 0.5\%$ 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 OPPu

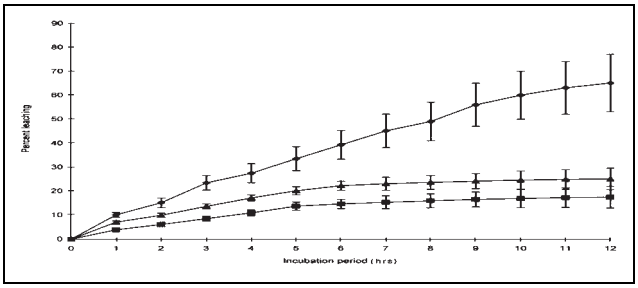


Fig. 2: Drug leaching studies in the simulated intestinal fluid (pH 6.8). Plain niosomes ●; OPPu coated niosomes ■; CHPu coated niosomes ▲

and CHPu 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 CHPu 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

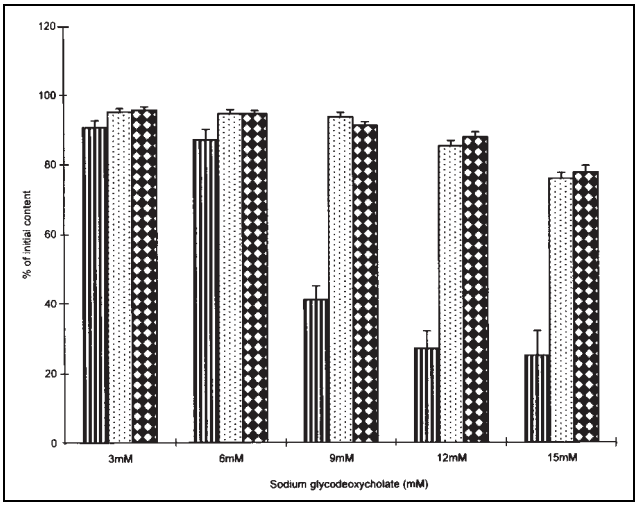


Fig. 3: Stability studies against stimulated bile salt solutions. Sodium glycodeoxycholate was added at increasing concentrations equivalent to the increasing values below, at and above CMC. Plain niosomes ▥; OPPu coated niosomes ⬚; CHPu coated niosomes ◆

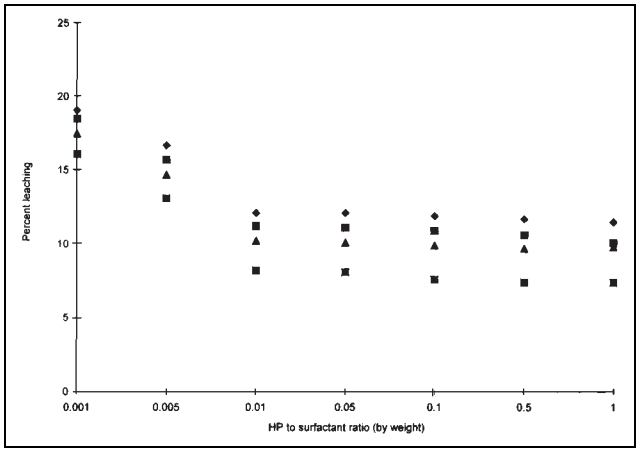


Fig. 4: Detergent stress studies in terms of drug leached (%) at different time of incubation. Incubation with detergent of OPPu coated niosomes for 60 minutes ◆ and 360 minutes ■; CHPu coated niosomes for 60 minutes ▲ and 360 minutes ■

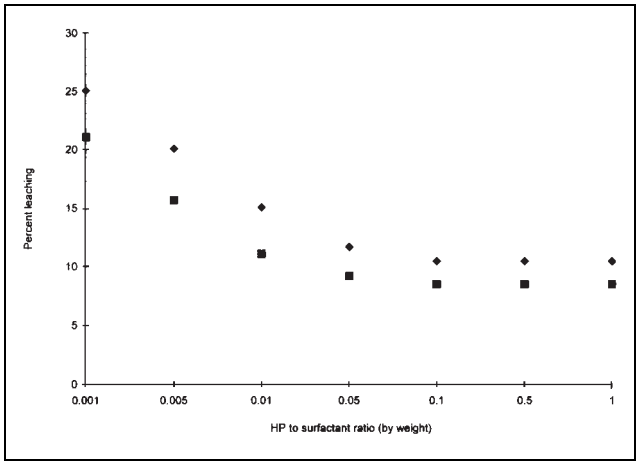


Fig. 5: Freeze-thawing studies in terms of drug leached (%). OPPu coated niosomes ◆; CHPu coated niosomes ■

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 Ucheghu and Vyas [9]. High concentrations of detergents are incompatible and cause eventual solubilization of the vescicles 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 underivatized pullulan (associated to niosomal membrane by adsorption) could not offer an appreciable protection to niosomes (not shown). For the earlier mentioned reasons, cholesteroylated 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 gradiance. 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

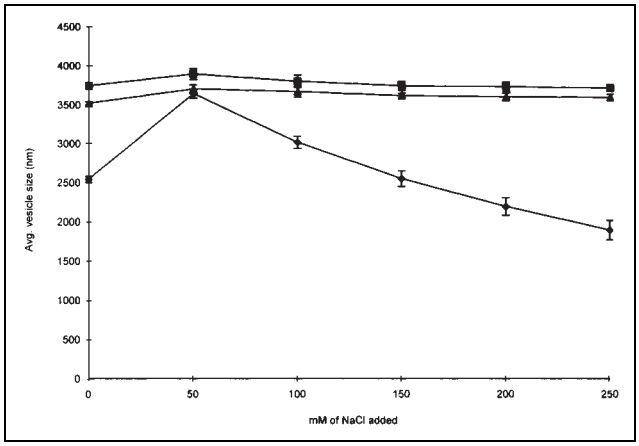


Fig. 6: Osmotic stress studies at different molar (hypotonic to hypertonic to plasma) concentrations of sodium chloride solutions. Plain niosomes ◆; OPPu coated niosomes ■; CHPu coated niosomes ▲

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 vesicles 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 $18.3 \pm 1.5\%$ leaching in the first 7 days, but in the next 21 days, niosomes degraded more rapidly leading to a $51.7 \pm 1.5\%$ leak after the first month. It was seen that vesicles lost their integrity and $89.1 \pm 4.1\%$ 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

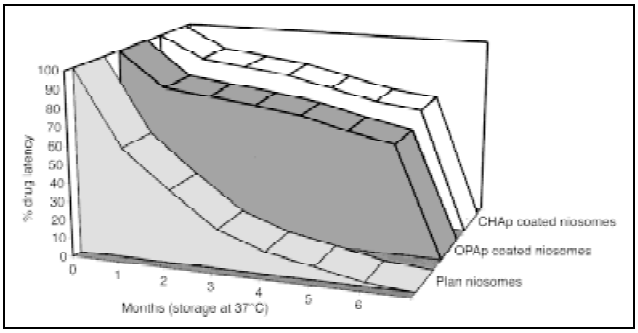


Fig. 7: Long term stability studies at physisologic temperature (37 ± 1 °C) estimated at different time intervals in terms of % drug latency

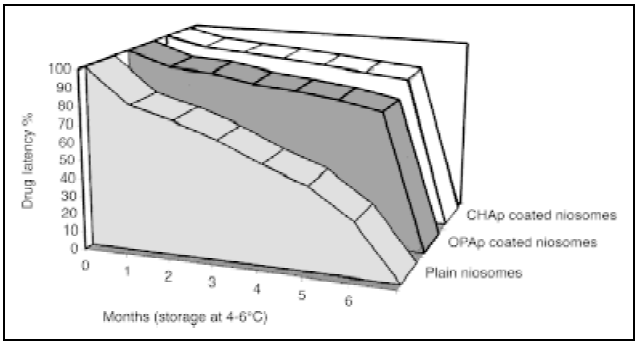


Fig. 8: Long term stability studies at physiologic temperature (4 ± 1 °C) estimated as the remaining drug per unit of surfactant per unit time (drug latency) at different time intervals

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-

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

Niosomal composition	Shelf stability in terms of % drug latency							
	1 week		1 month		3 months		7 months	
	37 ± 1 °C	4 ± 1 °C	37 ± 1 °C	4 ± 1 °C	37 ± 1 °C	4 ± 1 °C	37 ± 1 °C	4 ± 1 °C
Span 60 : CH (7 : 3)	−	±	−−	−	− − −−	− − −	− − −−	− − −−
Span 60 : CH : DCP (7 : 3 : 0.5)	±	+	−−	±	− − −	−−	− − −−	− − −
(Span 60 : CH : DCP) : CHPu (7 : 3 : 0.5) : 0.1	+	+	±	+	±	+	±	+
(Span 60 : CH : DCP) : OPPu (7 : 3 : 0.5) : 0.1	+	+	±	+	±	+	±	+

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%; (− − −) above 75%; (− − −−) vesicles disrupted and released their 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

Span® 60, cholesterol and dicetyl phosphate were purchased from Lobe Chemi, India. Pullulan from *Aureobasidium pullulan* 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 pullulan derivatives

Pullulan was derivatized with an O.palmitoyl anchor or a cholesterol moiety following the procedures of Hammerling and Westpal [17] or Sato [18]. O-Palmitoyl pullulan (OPPu), in brief, was prepared by treating pullulan (1.0 g) in dry DMF at 60 °C to 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 OPPu thus obtained was collected and washed with 50 ml of absolute ethanol and 25 ml of dry diethyl ether, and dried under vacuo at 50 ± 1 °C for 1 h (Scheme 1). The cholesterol derivative of pullulan was synthesized as described [2] with appropriate modifications. In brief, carboxymethylated pullulan was obtained by the reaction of pullulan (1.0 g) with sodium monochloroacetate (0.95 g) in 1 M NaOH. The resulting solution was treated at pH 4.7 with ethylenediamine (1.25 g) in the presence of 1-ethyl-3-(3-dimethylamino) propylcarbodiimide (0.5 g) as a coupling reagent. The aminoethyl carbamylmethyl pullulan thus obtained was reacted with cholesterol chloroformate (0.5 g) in a water free FMF solution at 60 °C for 24 h and the precipitate obtained was dried in vacuo at 50 °C (Scheme 2). The hydrophobized derivatives of pullulan (HP) were subjected to FT-IR and ^{1}H NMR analysis. The ^{1}H NMR spectrum was obtained in denaturated DMSO (50 μg/ml) containing tetrametylsilane (TMS) as internal standard operating at a frequency of 425 Mhz (Spectrometer Varion Unity-500). The IR spectrum of OPPu and Pu (1%), incorporated into a KBr disc, was run on a FT-IR single beam spectrometer (Carl Zeiss, Specord 75, Germany).

3.2.2. Preparation of niosomes

Span® 60, cholesterol and dicetyl 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 desiccating the mixture for about 1 h, 500 μl of PBS (pH 7.4) containing 10 μg/μl 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 capping 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 pullulan 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 pullulan 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 $\cong 5.1 \times 10^{-3}$ μg ml^{-3}). The dispersiosn mix was subsequently kept incubated in a microcuvette 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 capped vesicles

Polysaccharide coated niosomes were isolated by gel chromatographic separation. A Sephadex G-50 column was equilibrated with mixed phosphate buffers and pre-saturated with niosomal constituents (Length: 50 cm; diameter: 1.5 cm; flow rate: 150 μl/min; fraction volume: 2.0 ml/fraction). Fractions (2.0 ml elute) were collected and aliquots (100 μl) of the fractions were assayed for hydrophobized pullulan (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, Biomed, 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. Vesicles were lysed with 1% n-propanol : PBS (1 : 1 v/v), and centrifuged again. The liberated 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 uncoated and OPPu and CHPu coated niosomes (500 μl) were suspended in 2.5 ml of simulated gastric (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 290 nm for the released (leached) drug [25].

3.3.4. Detergent challenge and freeze thawing studies

Structural integrity of niosomal bilayers coated with hydrophobized pullulan was measured against the challenge of detergent (decyl-PEG) and freeze thawing. Drug loaded plain niosomes were pre-incubated with gradually increasing concentrations of hydrophobized pullulan for 60 min. Drug leaching was continuously estimated and decyl-PEG (0.05% w/v) was added. The rate of drug release from different hydrophobized pullulan 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 diluted with 0.05 M PBS (pH 7.4) to a final concentration of 0.5 mg 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 discussed earlier [25].

3.3.5. In vitro 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^{-1}. 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, Biomed, Germany) upon incubation with different molar solutions of sodium chloride. The stress study consists of incubation of small volumes (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 diluted appropriately and estimated for mean vesicle size.

3.3.7. 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 niosomal dispersions were removed, centrifuged at $60{,}000 \times g$ for 60 min and supernatant was analyzed 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 niosomes was checked (during storage) by their visual appearance with respect to aggregation and mean vesicle size, vesicles remaining per mm^3, and by measuring the leached drug as a function of time at experimental temperatures.

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