DEVELOPMENT OF LEISHMANIA DONOVANI ANTIGEN LOADED MANNOSYLATED BILOSMES TO TARGET M-CELLS: IN VITRO CHARACTERIZATION

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ABSTRACT

The aim of the study was to develop mannosylated bilosomes as oral vaccine delivery carrier for producing mucosal immunization against visceral leishmaniasis. Visceral leishmaniasis is the one of the most dangerous disease which may cause death if left untreated. The best solution of this problem is to develop immunization against the disease. Immunization can be produced via parenteral or oral routes but immunization produced by oral route is best suited and convenient for patient. Since bilosomes are prepared from naturally occurring lipids and produce no toxicity on their use, they can be used to replace the conventional lipid vesicular systems for the oral delivery of antigenic proteins. The ability of mannosylated bilosomes to produce immune response is higher than uncoated bilosomes and hence better oral immunization can be produced by using mannosylated bilosomes as carrier which are more stable in gastrointestinal tract. The bilosomes were prepared and coated with o-palmitoylemannan to enhance their affinity towards antigen presenting cells of peyer’s patches. The bilosomes were characterized for their size, shape, encapsulation efficiency, ligand binding specificity, antigen release, stability and targeting to M-cells of peyer’s patches. Mannosylated bilosomes were found to target the M-cells.

KEY WORDS: leishmania donovani, Mannosylated bilosomes, M-cells.
1. INTRODUCTION
Visceral leishmaniasis (VL) is a disease caused by *leishmania donovani* pathogen which is a flagellated pathogen (1). VL can be of two types- zoonotic and anthropopotic (2). Zoonotic VL is caused by *leishmania infantum*. In Zoonotic VL the pathogen completes its life cycle in animals like dogs and rodents and humans are only occasional hosts (3). *Leishmania donovani* is responsible for causing Anthroponotic VL. In Anthroponotic VL the pathogen passes from human to sand fly and then again to human (4). The cases of VL are found in whole world, 2 million new cases are found every year and 1/10 of the total world's population is at risk of infection. Hence it is one of the major causes of death worldwide which needs to be cure. Ninety percent of visceral leishmaniasis cases occur in Bangladesh, Brazil, India, Nepal, and Sudan. Untreated cases of kala-azar leads to 90% mortality, which can be reduced to 15% by treatment and even to 3.4% if proper treatment is given (5, 6). Visceral leishmaniasis is mainly caused by two species of leishmania pathogen that are *Leishmania donovani* and *Leishmania infantum*. *Leishmania infantum* is responsible for disease in immunocompromised persons and in children but *Leishmania donovani* can affect anyone. The vector for the pathogen is a sand fly i.e. Phlebotomine (1). The parasite involves two different forms during its life cycle that are promastigote and amastigote. The promastigote form is found in sand fly and the amastigote in the vertebrate host. The parasites after entering in dendritic cells and macrophages, gets converted into amastigotes. The amastigote multiply continuously inside the macrophages and ruptures them, liberating the amastigotes into the blood circulation. The free amastigote invades fresh cells, repeat the cycle and infect the entire reticulo-endothelial system (7, 8). The parasites scatter through the lymphatic and vascular systems and infect other monocytes and macrophages in the reticulo-endothelial system, resulting in accumulation of the bone marrow, hepatosplenomegaly and sometimes enlarged lymph nodes. Some of the free amastigotes are taken by the sandfly during its bite and thus completes the cycle (9).

Immunization or vaccination may prove to be more beneficial to reduce the occurrence of leishmaniam because the drugs available for visceral leishmaniasis are costly and have various serious adverse effects (10). Currently parenteral vaccines are more in use but these are having some side effects like pain on injection, need of trained personnel to administer the vaccine, patient inconvenience and risk of infections caused by the use of needle. Hence oral vaccination is preferable over parenteral route because it is safest, economical, mimics natural infection, higher mucosal immune response, ease of administration and strong first
line defense against pathogen (11). To deliver the antigen to the target site, different carriers can be used. Liposomes are bilayered vesicles that can be used to deliver the antigen to target site but these are affected by detrimental effect of bile salts in g.i.t and only a fraction of the total amount of antigen may be encapsulated within these liposomal vesicles that may cause inconvenience and increased cost (12). Similarly another carrier system i.e. niosomes also have to face disastrous effect of detergents (11). Nanoparticles are having very small size and less suitable for M-cell targeting because for targeting to these cells particles with size around 5µm are best suited (13). Bilosomes are vesicles prepared from non-ionic surfactants incorporating bile salts like sodium deoxycholate. These are resistant to degradation by gastric HCl and proteolytic enzymes (11). Hence among different carriers available like liposomes, niosomes and bilosomes; bilosomes are most effective as these remain stable against the detrimental effect of bile salts. As these are bilayered lipid vesicles, these resemble to the biological membrane (14, 15).

Macrophages and dendritic cells express mannose receptors, which are important in producing immune responses against the antigen as they play important role in phagocytosis, processing of antigen and their presentation as well as in intracellular signaling. The targeting to mannose receptors present on macrophages and dendritic cells can be done by mannosylation as mannose receptors are present on these cells. Hence mannose acts as a ligand for these receptors (16).

2. MATERIALS AND METHODS

2.1. Materials

Soluble Leishmania donovani antigen was a gift from CDRI, Lucknow and was used after dilution. Mannose was purchased from Ottokemi. Span 80 (sorbitanmonooleate) was purchased from SD Fine Chemicals Ltd, India. Palmitoyl chloride, Sephadex G-50 and G-100 and Concanavalin A were purchased from Sigma Chemicals Co. (USA). Cholesterol, sodiumdeoxycholate, bovine serum albumin were purchased from Hi-media Laboratories Pvt. Ltd. (India). All remaining chemicals and solvents were of analytical grade and purchased from local suppliers.

2.2. Preparation of BSA and Leishmania Antigen Loaded Bilosomes

Bilosomes are the bilayered lipid vesicles stabilized by bile salt and were prepared by film hydration method with some modifications (17). Span 80 and Cholesterol with 7:3 molar ratios were dissolved in 10ml chloroform in a round bottom flask. The chloroform was
evaporated by hand shaking method at a constant speed to form a thin film on the sides of flask. 3.5 ml of phosphate buffer saline (PBS, pH 7.4) was added to hydrate the film. The PBS containing sodium deoxycholate with a molar ratio similar to cholesterol along with BSA and *leishmania donovani* antigen was used for preparing BSA loaded and Leishmania antigen loaded bilosomes respectively. The total volume of the preparation was made upto 5 ml with PBS. The unentrapped sodium deoxycholate and antigen were removed by passing the formulation through Sephadex G-100 mini-column and centrifugation at 3000 rpm for 3 mins.

2.3. Synthesis and Characterization of O-palmitoylmannan
O-palmitoylmannan was synthesized from mannose by esterification process. Two gram of mannose was reacted with 0.2 g palmitoyl chloride in the presence of 100 ml dry dimethyl formamide. Followed by 2 ml dry pyridine was added under anhydrous conditions and stirring was continued for 2 hrs at 60°C and then at room temperature for 3 hrs. The precipitates of OPM were collected, washed with absolute ethanol and dry diethyl ether. The precipitates obtained were dried at room temperature. The OPM obtained was characterized by FT-IR Spectroscopy (18).

2.4. Coating of O-palmitoylMannan (OPM) on Bilosomes
The coating of bilosomes was done by taking 2 ml of uncoated bilosomal formulation having encapsulated leishmania antigen. The OPM solution in PBS (pH 7.4) was prepared by taking lipid: ligand ratio 5:1 in sufficient quantity of PBS. The formulation was then incubated with OPM solution. The mixture was stirred gently at room temperature for 4 hrs. The suspension was passed through sephadex G-50 column at 3000 rpm for 5 mins to remove the unbound polysaccharide (19). Similarly, BSA loaded mannosylated bilosomes were also prepared. BSA loaded formulation was used to study the effect of variables on formulation.

2.5. Characterization of bilosomes and mannosylated bilosomes
The formulations were characterized for shape, size and morphology by transmission electron microscopy using 0.2% phosphotungustic acid solution (20). The vesicle size and polydispersity index were carried out by Zeta seizer. The surface charge was also determined by Zeta potential measurement (21).

2.6. *In vitro* ligand binding specificity
The OPM coated bilosomal formulation was characterized for *in vitro* ligand binding specificity. Coated and uncoated bilosomal formulation (0.2 ml) were taken and diluted with
PBS (pH 7.4). This content was reacted with 1 ml of concanavalinA (1 mg/ml) in PBS (pH 7.4) having 5mM of calcium chloride and 5mM of magnesium chloride. The increase in turbidity was measured at 550 nm spectrophotometrically after 2 hrs (21).

2.7. In vitro antigen release study
The in vitro antigen release of encapsulated antigen bilosomal formulations were studied by using dialysis bag method. Accurately measured coated liposomal formulations (2ml) were taken into a dialysis bag (Sigma, USA) and placed in a beaker containing 50 ml of PBS (pH 7.4). The beaker was placed on a magnetic stirrer at 25 ± 1°C. Aliquots were withdrawn after 1, 2, 4, 8, 10, 12, 24, 48, 72, 96, 120 and 144 hrs time intervals and replaced with the same volume of PBS. The aliquots were assayed for antigen content by BCA protein assay method (22).

2.8. Stability studies in simulated gastric fluid
The stability studies of bilosomal formulation were carried out in simulated gastric fluid (SGF) and simulated gastrointestinal fluid (SIF). The formulations were diluted with SGF and SIF of pH 1.2 and 6.8, respectively. The formulations were shaken with the gastric fluids for 2 hours using shaking incubator at 36°C. Significant change in vesicles size was observed (21). The effect of temperature on vesicle size and consistency was also studied by keeping the formulation at 4±1°C and 25±2°C, 60±5% RH.

2.9. Fluorescence microscopy
The deposition of bilosomes in GALT cavity of macrophage was confirmed by using fluorescence microscopy. Coumarin-6 fluorescent dye was used as a fluorescent marker. The bilosomes were loaded with the dye. The formulation was administered to the mice with the help of a canula. Animals were sacrificed, after 5 hrs of administration of the formulation, the small intestine was removed, cut and microtomy was performed. The sections of about 3 µm thickness were viewed under the fluorescence microscope. Equivalent amount of unentrapped coumarin-6 (5 mg in 500 µl PBS (pH 7.4)) was given orally to the control groups and microtomy was performed (20).

3. RESULTS
3.1. Preparation and in vitro characterization
The method used for the preparation of bilosomes was film hydration method. The components used for the preparation were sorbitanmonoooleate (span 80), cholesterol and bile
salt. The effect of lipid to lipid molar ratio was studied by preparing different batches from F1 to F3 with different molar ratio of span 80 and cholesterol (6:4, 7:3, 8:2) and other parameters like bile salt molar ratio (1), antigen concentration (10 µg/ml), sonication time (3 mins) were kept constant. The encapsulation efficiency of F2 batch was maximum 51.23 ± 1.20% and the mean vesicle size and polydispersity index were 0.171±0.19 µm and 0.213±0.03, respectively(Figure 1).

To study the effect of bile salt ratio, formulations from F4 to F6 having different bile salt ratio (7:3:2, 7:3:3, 7:3:4) were prepared. The maximum antigen encapsulation was found in span 80: cholesterol: bile salt in the ratio of 7:3:3 that was 44.6± 1.21% and the mean vesicle size and polydispersity index were 0.131±11µm and 0.161±0.9, respectively(Figure 2).

The effect of antigen concentration was studied by preparing different batches from F7 to F9 with antigen concentration (10, 20 and 30µg/ml) while all other parameters were kept constant. The batch with 20µg/ml antigen concentration showed maximum encapsulation efficiency 43.5±1.22%, mean vesicle size was 0.213±21µm and polydispersity index was found to be 0.179±0.7(Figure 3).

The best formulation was selected and subjected to sonication for different time periods (4, 5, 6 mins) to study the effect of sonication time by preparing batches from F10 to F12 with constant lipid to bile salt ratio (7:3:3) and antigen concentration (20 µg/ml). The encapsulation efficiency was increased from 34.89 ± 1.22% to 47.83 ± 2.43% and then decreased(Figure 4).

Mannosylated bilosomes were evaluated to study the effect of lipid to ligand (OPM) ratio and incubation time. To evaluate the effect of lipid to ligand ratio, F13 to F 15 batches were prepared and were coated with different ratio of OPM (4:1, 5:1, 6:1) keeping all other parameters constant. The zeta potential was decreased from -40 to -63.2 mV(Figure 5).

To study the effect of incubation time, F16 to F19 batches were prepared and incubated for different time periods (3, 4, 5 hrs) keeping all other parameters constant. The change in zeta potential was observed. The zeta potential decreased from -47.9 to -68.9 in F16 to F17 batch. The zeta potential value showed a slight decrease in F17 to F18 batch from -68.9 to -71.2 (Figure 6).
From the above optimized parameters, the encapsulation efficiency of leishmania antigen loaded mannosylated bilosomes was found to be 46.25±1.19% and the mean vesicle size and zeta potential were 0.208±13µm and -55mV, respectively (Table 1). The optimized final formulation was selected for further studies.

The prepared final bilosomes formulations were characterized for their shape, morphology, vesicle size, size distribution, number of vesicles and in vitro ligand binding specificity. The bilosomal formulations were characterized for their shape and morphology by transmission electron microscopy (HRTEM, Hitachi H 7500) as shown in Figure 7. The size of the bilosomes was determined by zeta sizer (Malvern Instruments, UK) and shown in Figure 8. Surface charge of the bilosomal formulations were determined by Zeta potential measurement and shown in Figure 9.

3.2. In Vitro ligand binding specificity
The coating of mannose on bilosomal surface was confirmed spectrophotometrically by using concanavalinA agglutination assay at 550 nm. The results are graphically represented in Figure 10.

3.3. In vitro Antigen Release Study
The in vitro antigen release study of mannosylated bilosomes were carried out for 144 hrs in PBS (pH 7.4). It was observed that the antigen release was 83.12±0.00002 after 144 hrs. The finalized batch was carried out for in vitro drug release studies for 144 hrs in phosphate buffer (pH 7.4) by dialysis bag technique using dialysis membrane. In vitro release rate of leishmania antigen loaded mannosylated bilosomes was graphically presented in Figure 11. The cumulative % antigen release in the 24 hrs, 72 hrs, 120 hrs and 144 hrs, was 41.98±0.00001%, 63.42±0.00009%, 77.23±0.00007% and 83.12±0.00002%.

3.4. Stability studies in SGF and SIF
The stability of the formulation was determined in simulated gastric fluid (SGF, pH 1.2) and simulated intestinal fluid (SIF, pH 6.8). The results showed that after 2 hrs no significant change in vesicles size was observed.

The effect of temperature on vesicle size and consistency was studied by keeping the formulation at 4±1°C and 25±2°C, 60±5% RH. The results are graphically represented in Figure 12 & Figure 13, respectively.
3.5. Fluorescence Microscopy

The targeting of coated bilosomes to GALT cavity of M cells was confirmed by fluorescence microscopy as shown in Figure 14.

**Figure 1: Effect of Span 80 to Cholesterol Ratio**

**Figure 2: Effect of Lipid to Bile Salt Ratio**
Figure 3: Effect of Antigen Concentration

Figure 4: Effect of Sonication Time

Figure 5: Effect of Lipid to Ligand Ratio on Mannosylation
Figure 6: Effect of Incubation Time on Mannosylation

Figure 7: TEM image of leishmania antigenmannosylated bilosomes

Figure 8: Zeta sizer of leishmania antigen loaded loadedmannosylated bilosomes
Figure 9: Zeta potential of Leishmania antigen loadedmannosylated bilosomes

Figure 10: *In Vitro* Ligand Binding Specificity of uncoated and coated Bilosomal formulations

Figure 11: *In vitro* antigen release of uncoated and coated Bilosomal formulations
Figure 12: Stability Study of leishmania antigen-loaded mannosylated bilosomal at formulation at 4±1°C

Figure 13: Stability Study of leishmania antigen-loaded mannosylated bilosomal formulation at 25±1°C, 60±5% RH
Table 1: Various parameters of finalized BSA loaded mannosylated bilosomal formulation

<table>
<thead>
<tr>
<th>Formulation</th>
<th>Mean Vesicle Size* (µm) ± S.D.</th>
<th>Zeta Potential (mV)</th>
<th>% Encapsulation Efficiency* (%w/w) ± S.D.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Leishmania antigen loaded Mannosylated Bilosome</td>
<td>0.208±0.13</td>
<td>-55.0</td>
<td>46.25±1.19</td>
</tr>
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*(Mean ± S.D.) (n=3)

4. DISCUSSION

The lipid to lipid molar ratio of 8:2 showed lower encapsulation efficiency due to vesicle lysis. It may be due to the decreased concentration of cholesterol which provides strength to the vesicles. The encapsulation efficiency at 7:3:4 was lower due to high concentration of sodium deoxycholate. At high concentration sodium deoxycholate shows emulsifying property on cholesterol which decreases the rigidity of vesicles. As the sonication time was increased from 4 min to 5 min, the average vesicle size decreased. On further increasing the sonication time to 6 mins, the aggregation of lipid vesicles occurs due to lysis and release of lipid of some vesicles. While studying the lipid to ligand ratio, the addition of anionic natured OPM as ligand, zeta potential approached towards a minimum value at 5:1 lipid to ligand weight ratio because OPM mask the surface charge. On further addition of OPM only a slight decrease in zeta potential occurred i.e. no significant change. It indicates that at 5:1 lipid to OPM ratio, the interaction of OPM with the surfactant bilayer membrane occurs at saturation level. The zeta potential value decreased due to charge quenching of surface associated free OPM while studying the effect of incubation time. After 4 hrs, the residual free OPM
decreased and further change in zeta potential was not significant after 5 hrs. This shows that at the end of 4 hrs, the interaction of added OPM could have completed.

TEM image of the formulation showed that the vesicles were smooth, spherical, morphologically similar and uniformly distributed without any aggregation. The zeta seizer indicated the size of bilosomal vesicles. The negative value of zeta potential revealed the coating of o-palmitoylmannan which is negatively charged.

During in vitro ligand binding study, significant increase in turbidity was observed in case of mannose coated bilosomes while uncoated bilosomes didn’t show significant increase in turbidity. The increase in turbidity in case of coated bilosomes was due to release of coated mannose in formulation on reacting with concanavalin A.

To explore and explain the mechanism of antigen released from the mannosylated bilosomes, formulations were subjected to the various release kinetics. It was observed that the formulation followed the korsemeyer-peppas kinetic model when leishmania antigen was loaded in mannosylated bilosomes (not shown). This model described the sustained release of antigen from formulation and the mechanism of release was erosion and swelling leading to diffusion for antigen release from mannosylated bilosomes.

The formulation showed no significant changes in SGF (pH 1.2) and SIF (pH 6.8) after 2 hrs which indicates that the formulations can withstand pH changes of g.i.t. The changes in the size of different formulations were observed at 4±1°C and 25±2°C, 60±5% RH. When mannosylated bilosomal formulation was stored at 4±1°C, the size increased from 0.208.5±3 μm to 0.302.7±8 μm i.e. no significant change in the size of the formulation was observed after 3 months. The formulation when stored at 25±2°C, 60±5% RH showed significant difference in the average particle size as the particle size increased from 0.208.5 μm to 0.357.7 μm after 3 months. This may be due to aggregation of vesicles and degradation of the phospholipids at higher temperature. The consistency of the formulation remains unaltered at 4±1°C while the consistency was increased when the formulation was stored at 25±2°C, 60±5%.

The photograph of peyer’s patches of mice to which dye was administered, showed that the dye was unable to target the cells as the fluorescence occurs outside the cells but when dye was administered to leishmania loaded mannosylated bilosomal formulation, it showed
accumulation in the cells which was confirmed by fluorescence inside the cells due to coumarin 6 in formulation. It showed that mannosylated bilosomes can be used for targeting the antigen to M cells. The targeting to M cells showed that the formulation can prove to be beneficial to produce oral immunization against the deadly disease visceral leishmaniasis.

5. CONCLUSION
The purpose of the study was the development of mucosal immunization against visceral leishmaniasis using mannosylated bilosomes via oral route to reduce the side effects of conventional drugs and to overcome the limitations of parenteral route and to prevent the occurrence of visceral leishmaniasis. Using film hydration method uncoated bilosomes were prepared which can be potentially useful for the delivery of antigen. The targeting of the formulation could be done by mannosylating the uncoated formulation with OPM. The formulation was found to be quite stable at 4 ±10°C. From in vitro drug release study, it was concluded that the release of antigen from the formulation followed sustained release. It can be concluded from the result obtained that mannosylated bilosomes facilitate the oral delivery of antigen to the macrophages of liver, spleen through M cells and could be able to produce immunization against visceral leishmaniasis. Thus, the results of the present investigation showed that the problems associated with the treatment of visceral leishmaniasis could be overcome by using immunization method for prophylaxis and treatment of visceral leishmaniasis.

6. ACKNOWLEDGEMENT
I would like to acknowledge the support of CDRI, Lucknow for providing the gift sample of *leishmania donovani* antigen. I also want to sincerely thank to Mr. S.K Saniyal to perform the fluorescence microscopy study in Panjabi University, India.

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