

LIPOSOMAL MEDIATED ANTIGEN DELIVERY: ISOLATION AND CHARACTERIZATION

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Abstract

Objective: The present study was aimed on developing and characterizing Liposomal Delivery System loaded with antigen; filaria parasite extracted protein for better sustain release antigen profile.

Methods: Liposomes were prepared by Reverse Phase Evaporation (REV) method (Szoka and Papahadjopolus; 1978) with slight modification using molar ratio of Soya PC:PE:Cholesterol in different molar concentration.

Results: In the present study the storage stability of the vesicles was determined by measuring the vesicle size, residual antigen content and antigen integrity before and after 30 Days at $4\pm 1^\circ\text{C}$ and $25\pm 1^\circ\text{C}$. In Soya PC:PE:CH liposomes only a slight increase in size on storage at $25\pm 1^\circ\text{C}$ and insignificant change in size on storage at temperature $4\pm 1^\circ\text{C}$ occur, indicate that Soya PC:PE:CH liposome are more stable than conventional Soya PC:CH liposomes. Percent of residual antigen remain in liposomes by assuming the initial content to be 100%, in Soya PC:PE:CH liposomes only 14-15% antigen was lost at temperature $25\pm 1^\circ\text{C}$ and 5-6% antigen was lost on storage at $4\pm 1^\circ\text{C}$. Antigen integrity was evaluated by performing the SDS -PAGE of the liposome formulations. (Optimized CL3 formulation stored at $4\pm 1^\circ\text{C}$) after 30 Days. Antigens were found to be intact in the formulation stored at $4\pm 1^\circ\text{C}$ after 30 days. There was no effect of storage on structural integrity of the antigen (fig; 9.5B and fig; 9.5C).

Conclusion: These results suggest that the liposomal antigen delivery system is promising carriers for antigen delivery and vaccine development.

Keywords: Antigen, Filaria Parasite, BmAFII, Isolation of Antigen, B. malayi, Soya PC:PE:CH liposomes.

1. INTRODUCTION

Lymphatic filariasis (LF) commonly known by the name elephantiasis is a mosquito-borne tropical disease caused by the filarial nematodes *Wuchereria bancrofti*, *Brugia malayi* and *Brugia timori*, elicits a wide spectrum of pathological disorders of the lymphatic system with varied clinical manifestations. The filarial parasites can survive in the human for many years causing permanent disability due to chronic syndromes such as lymphoedema, elephantiasis, and hydrocoele. The World Health Organization (WHO) ranks it as the second most common cause of long-term disability and estimated that over 1.25 billion people are at risk of the infection in 83 countries and territories (WHO, 2006a). Approximately 125 million already have been infected with LF, and over 40 million (WHO, 2004a) are seriously incapacitated and disfigured by the disease (Ottesen et al., 1997). In 2002, it was estimated that LF is responsible for the loss of 4.52 million disability adjusted life years (DALYs) in men and over 1.42 million DALYs in women (WHO, 2004b).

The methods to control and prevent the filarial infection include administration of antifilarials alone or combination of diethylcarbamazine (DEC)/ivermectin and albendazole and exposure control programs. In recent years, identification of several filarial antigens/proteins or molecules raised hopes for developing vaccines (Gregory et al., 2000; Ramachandran et al., 2004; Krithika et al., 2005; Babayan et al., 2006; Vedi et al., 2008; Sahoo et al., 2009; Shakya et al., 2009; Joseph et al., 2012) against lymphatic filariasis.

Novel adjuvants have been developed for enhancing antigen delivery and reducing the vaccine delivery to a single injection. For future human use, it is however necessary to use an adjuvant that is safe, biodegradable and which does not require repeated administration to produce the desired result.

Filarial parasites present a diverse array of antigens which elicit a complex and broad spectrum of immune and inflammatory responses in their hosts. Some of the responses are suppressive, some are protective and some are irrelevant. Several attempts have been made to identify and correlate the host immune responses with parasite antigens, but in the small number of experimental systems, such correlations remained inconclusive. Many host responses, both protective and non-protective, have the potential to cause pathological changes both locally and systemically and this, poses problems for the development of immuno-prophylactic strategies. Where pathology is associated with irrelevant responses, it may be possible to devise approaches that elicit only desirable protective responses. However, when protection and pathology reflect facets of the same response, the problems are much more complex to handle. Thus, to overcome such problems, precise identity of the molecules and delineation of the immune mediated pathways activated by the parasite molecules are prerequisites.

Studies conducted in laboratory revealed that of the two major Sephadex G-200 eluted fractions of *B. malayi* adult worm extract, BmAFII is protective *in-vivo* and stimulates predominantly pro-inflammatory cytokines to both adult worms and L3 while BmAFI facilitates parasite survival and stimulates predominantly IL-10 release (Dixit et al., 2004; Dixit et al., 2006). Further, to narrow down to molecular entities that have cytokine release stimulating potential, *B. malayi* adult worm extract was fractionated by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and the resolved fractions were blotted onto NCP and screened for the cytokine release potential. Some NCP-bound molecules corresponding to the molecular weight range of BmAFI and BmAFII, were found to possess proinflammatory and mixed pro- and anti-inflammatory cytokine stimulating potential *in-vitro* and provided a starting point for precisely identifying the functional molecules/proteins of interest (Dixit et al., 2004).

Based on the above findings 6 molecular fractions (F1, F2, F5, F6, F10 and F14) were studied and F6 was selected for the present study to find out which of these fractions have the molecules potentially relevant to host protection or filarial pathology. The present work was aimed at characterizing parasite molecules with respect to their immunogenicity, involvement in the pathology of filariasis and to check its potency via novel carrier delivery system.

Liposomes have been used in drug delivery for many years. Liposomes, discovered in 1965 by Bangham and his colleagues are spherical colloidal particles containing an aqueous core surrounded by phospholipid bilayer which replicates cell membrane. Liposomes are microscopic lamellar structures formed on the admixture of soya lecithin with subsequent hydration in aqueous media. Liposomes have been widely evaluated for controlled and targeted drug delivery for treatment cancer, viral infections and other microbial diseases.

Liposome vaccines have several advantages, including sustained release, prevention of local irritation, reduced toxicity, improved stability in the large aqueous core, and the possibility to manipulate release and targeting by altering the bilayer constituents and changing the preparation technique. The drug carrying capacity, release rate, and deposition of liposomes is dependent on the lipid composition, size, charge, drug/lipid ratio, and method of delivery. Conventional liposomes are composed of neutral or anionic lipids (natural or synthetic). The most commonly used are the lecithins (phosphatidylcholines), phosphatidylethanolamines (PE), sphingomyelins, phosphatidylserines, phosphatidylglycerols (PG), and phosphatidylinositols (PI). Dry powder liposomes have been produced by lyophilization followed by milling, or by spray-drying.

There is an immediate need for the development of new and improved adjuvant and delivery system, which are potent, safe and can be used as a new generation vaccine. In the present study it is thought worthwhile to prepare liposomal-system having the potential benefits of reducing the number of dosages for primary immunization, reducing the total antigen dose required for effective immunization, enhancing both humoral and cell-mediated immune responses over a longer period of time, enabling combined vaccine administration and permitting effective primary or booster immunization. In addition, the integrity of the antigen is maintained by

avoiding the use of organic solvents and a pH changes, preparation process is simple and easy to scale up for chemical studies and eventual manufacture.

The surface antigen(s) have important role in generation of protective immunity. Consequently, characterization of protective responses generated by surface antigen(s) that can be used as vaccine is worth considerable. A few body wall antigens have earlier protective and are potential vaccine candidates against filarial infections. The purified native protein or recombinant filarial protein might be more useful for achieving the desired immunity.

Therefore, the present study was aimed to isolate the purified native protein of parasite and to prepare novel liposomal system in optimized ratio which would enhance desired immune response with minimum toxicity and characterize the prepared dosage form in terms of size, shape and adsorption capacity to determine optimum dose and also assess immunoadjuvancy of prepared dosage form in antigen dose reduction by using different immunization protocols.

Materials and methods

The pilocarpine HCl was kindly received as a gift sample by M/s Zydus Cadila Health Care Ltd. (Ahmedabad, India). Sorbitan monolaurate (span 20), sorbitan monoesterate (span 60), sorbitan monooleate (span 80), cholesterol, locust bean gum and carbopol 934 were procured from Loba Chemie Pvt. Ltd. (Mumbai, India).

Antigen isolation

Parasite

For laboratory experimental purpose, *Brugia malayi*, having many of the biological aspects similar to *Wuchereria bancrofti*, was used in the study. It is a sub periodic strain of human filarial infection and has successfully been transmitted to various vertebrate hosts including monkeys, cats and rodents. The infection is transmitted through black eyed susceptible strain of *Aedes aegypti* mosquitoes developed by McDonald (Liverpool School of Tropical Medicine and Hygiene, U. K.).

Animal models

Rodents are preferred models for laboratory studies throughout the world. 'GRA' strain (Germany) of *Mastomys coucha* (belonging to family Muridae) as shown in Figure 1 is susceptible to *B. malayi* and is being maintained in the animal house of Central Drug Research Institute, Lucknow, India since last 35 years. It is a multimammate, prolific breeder with average litter size of 8 - 10 babies. The female may have her young at any time of the year and if conditions are right, may do so regularly at intervals of 33 days (Wilson and Reeder, 1993). *M. coucha* model is found amenable to perform chemotherapeutic and immunobiological investigations in experimental filariasis (Mukhopadhyay et al., 1996; Tyagi et al., 1998). Keeping in view their similarity of immune responses to human, this animal is used as model for experimental purpose (Dixit et al., 2004; Gaur et al., 2007; Khan et al., 2004).

Another rodent, the Mongolian gerbil (*Meriones unguiculatus*, family Gerbillinae) as shown in Figure 1 has proven to be an excellent permissive rodent model for the study of lymphatic filariasis using *B. pahangi* or *B. malayi* (McVay et al., 1990). The animal (called 'jird') is used for the propagation of *B. malayi* parasites (McCall et al., 1973).

All the experiments were conducted in compliance with the Institutional Animal Ethics Committee guidelines for use and handling of animals. The animals are kept in plastic cages and were housed in animal quarters under controlled climate (23 ± 2 °C; RH: 60%) and photoperiod (12 h light-dark cycles). They were fed with standard rodent diet pellets and had free access to drinking water.

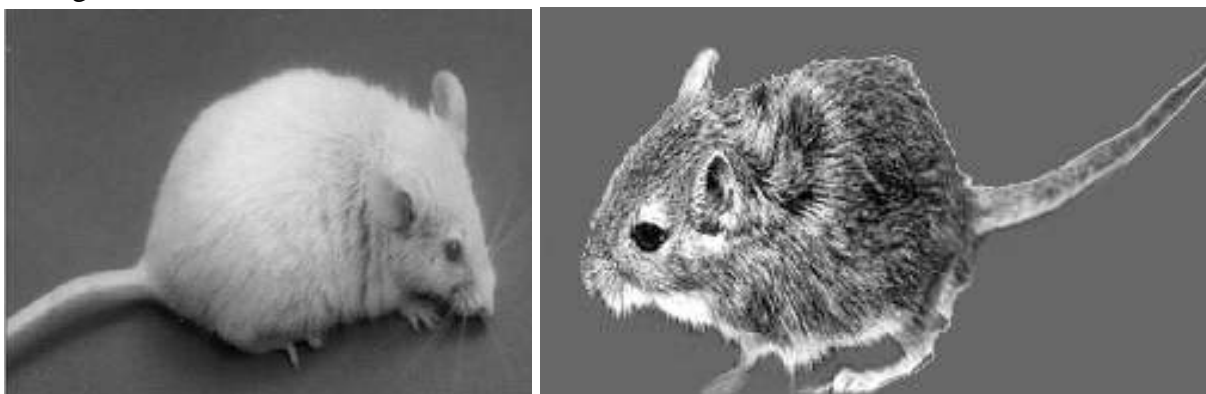


Fig. 3.1: Experimental Filarial models – *Mastomys coucha* & *Meriones unguiculatus*.

Maintenance of *B. malayi* infection

Rearing and breeding of *A. aegypti* colony

In the laboratory the mosquitoes were reared and bred in an insectarium maintained at controlled temperature (26 ± 1 °C) and humidity (80 ± 5 %). The adult mosquitoes were kept in nylon mesh cages and provided 10% glucose solution with vitamin B supplement, soaked in cotton for feeding. From time to time female mosquitoes were fed on normal *M. coucha* blood to promote egg laying. A beaker containing water was kept in the cage for egg laying. The eggs laid after about 40 hrs blood feeding were filter separated and stored after drying at same temperature. Eggs can be preserved under such condition for 3 - 4 months. For maintenance of mosquito life cycle the eggs were transferred to enamel bowl containing tap water. The larvae hatch out in the water within 24 hrs and these were provided with feed containing dog bix and yeast powder. The larvae usually took about 8-10 days to become pupae, which ultimately developed into adult mosquitoes within 48 hours.

Feeding of mosquitoes on mf positive *M. coucha*

B. malayi infected *M. coucha* showing 100-200 mf/10 µl of blood were used as donors. The feeding of mosquitoes on donors was carried out between 12 noon and 1:00 PM (peak microfilaraemia time). Mosquitoes starved for 2-3 hrs were allowed to feed on the donors, which

was kept inside the mosquito cage in a wire netting immobilized cage. After 1 hr of feeding the donor animal was removed and mosquitoes were provided with glucose solution as mentioned above. In 9 - 10 days' time the mf in the mosquitoes developed into L₃.

Isolation of L₃ from mosquitoes

On day 9 or 10 post feeding, the mosquitoes were paralyzed and crushed gently in 4-5 ml of 0.6% insect saline (IS) and transferred to Baerman's apparatus which consisted of glass funnel, muslin cloth and transparent rubber tubing with a pinch cock. The funnel was filled with lukewarm IS. Crushed mosquitoes were then put onto muslin cloth and allowed to stand for half an hour with light provided from top by a table lamp. The L₃ released from the mosquitoes move away from light, traverse through the muslin cloth and settled down at the bottom of the tube. These were collected by opening the pinchcock and washed with IS several times to remove the mosquito debris. The larvae were counted and used immediately for exposure to animals.

Inoculation of L₃ to *M. coucha* or jird

For infection purpose 6-8 weeks old male *M. coucha* were inoculated with active and motile L₃ (100 per animal) subcutaneously. Establishment of successful infection was examined in blood smear after day 90 post larval inoculation and thereafter monitored at regular intervals. Animals showing desired levels of infection were used for transmission to healthy animals. Thus, the cycle was continued. Similarly in jirds of the same age group about 200 L₃ were inoculated intraperitoneally. The larvae develop into adult worms in about three months and can be harvested thereafter when required.

Preparation and fractionation of antigen

The worms were washed several times and crushed in phosphate buffered saline (PBS) in cold followed by sonication (Soniprep 150) for 10 cycles of 30 seconds each at 10 micron amplitude with intermittent gap of 1 minute. The total homogenate was mixed with equal amount of 2X sample buffer for sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS PAGE) (0.125 M Tris-HCl, pH 6.8; 4% SDS, 5% β-mercapto ethanol, 20% glycerol and 0.01% bromophenol blue) and boiled in water bath for 5 min. The protein samples thus prepared were centrifuged to remove any particulate residue before loading to gel.

Sodium dodecyl sulphate -polyacrylamide gel electrophoresis

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) is the most widely used analytical method to resolve separate components of a protein mixture. SDS-PAGE simultaneously exploits differences in molecular size to resolve proteins differing by as little as 1% in their electrophoretic mobility through the gel matrix.

In order to isolate dominating fractions which have been identified to be stimulators of pro- and mixed pro- and anti-inflammatory cytokine release (Dixit *et al.* 2004; Table 3.1) extract of adult worms was used. The extract was resolved in 10% gels (Laemmli, 1970). Preparative (13.8×13

cm slab) gels were run in gel with a dual-gel electrophoresis chamber (AE-6220, Atto Japan). Resolved fractions (six) of interest were cut with the help of pre-stained molecular weight markers run along the side. These were designated as F1, F2, F5, F6, F10 and F14. The fractions in gel strips were stored as such in gel at -10°C till elution.

Table 3.1: Cytokine-release stimulating potential of NCP-bound molecules of adult *Brugia malayi* soluble extract in THP-1 cell system

Fraction (MW kDa)	Cytokine
F1 (>180)	TNF- α (++) , IL-10 (++)
F2 (169-180)	TNF- α (+++) , IL-10 (+++)
F5 (67.8-84.3)	IL-1 β (+++)
F6 (54.3-67.8)*	IL-1 β (+++), IL-6 (+++), TNF- α (+)
F10 (38.44-41.84)	IL-1 β (++)
F14 (17.0-22.5)	IL-10 (+)

+, ++ & +++ indicate ascending grade of predominance.

* selected for present study.

Electro-elution of proteins from gel

Proteins from gel strips were electro-eluted by micro electro eluter (Millipore, USA) as per method described by manufacturer. Briefly, about 75 % of the perforated tube was filled with SDS PAGE gel strips in small pieces. The tube carrying gel was fitted into centricon™ tubes having a membrane filter of required cut off limit. After filling both upper and lower chambers of the microeluter with Tris-glycine buffer, electricity (~200V) was applied for 2 to 4 hrs depending on the size-based mobility of the fraction ensuring near complete elution from the gels. After elution is over the centricon tube was disassembled from the slot and the gel carrying tube was removed. Protein solution retained in the centricon was centrifuged at 2000g in cold (4 °C) in an angular rotor till the volume reached to required level.

The eluted fractions were run in SDS-PAGE to confirm their molecular weight. Finally the protein solution thus obtained was filter sterilized with 0.22 μ membrane filter and stored at -20°C until used.

ANTIGEN SELECTED FOR STUDY**Bovine serum albumin (BSA) as a model antigen**

BSA is a white to light tan colored powder that contains not more than 3.0% w/w of water, containing about 96 % protein. It has a molecular weight of 67KDa. It consists of a carbohydrate free polypeptide chain connecting four globular segment of unequal size. It must be protected from light and moisture and store at temperature between 2° C and 25° C.

***Brugia malayi* adult worm protein extract (F6) as a candidate antigen**

F6 is a sephadex G-200 eluted fraction of *B. malayi* adult worm extract. It has a molecular weight of 54.3-67.8 KDa. It has five proteins namely heat shock protein (HSP60), NAD dependent epimerase/dehydratase, intermediate filament (IF), elongation factor 2 (EF2), hypothetical protein CBG00623. It must be protected from light and moisture and store at temperature -20°C.

PREPARATION OF LIPOSOMES

Liposomes were prepared by reverse phase evaporation (REV) method (Szoka and Papahadjopolus; 1978) with slight modification using different molar ratio of Soya PC: PE: Cholesterol. PC: PE: CH was dissolved in 5ml of diethyl ether. To the above solution, 3ml of phosphate buffer saline (PBS) pH 4.2 was added. The mixture was sonicated for 1.0 minute at 4°C. The mixture was kept in a rotary vacuum evaporator; the organic solvent was removed under vacuum (260 to 400 mm Hg) at 37±1°C until it became thick. The gel was then subjected to vigorous mechanical agitation on a vortex mixture to form a suspension of liposomes. Various liposomal formulations with increasing concentration of PE were prepared.

Table 1. Composition for liposomes.

S. No.	Formulation Code	Ratio (PC: PE: Chol)
1	CL1	8:0:2
2	CL2	7.2:0.5:2
3	CL3	7.0:1.0:2
5	CL4	6.6:1.4:2
6	CL5	6.2:1.8:2

Table 3. Evaluation of niosomes.

Formulation Code	Entrapment efficiency (%)	Vesicle size (µm)	Zeta potential (mV)	Q8h (%)
F1	81.43±2.09	1.44±2.76	-31.04±0.25	78.81±4.82
F2	86.17±3.07	1.56±1.59	-29.54±0.72	73.15±6.32
F3	78.56±0.99	2.71±0.57	-28.84±0.27	68.74±0.78
F4	93.26±1.75	6.13±0.31	-15.04±0.45	60.35±1.87

F5	90.66±3.80	5.16±0.27	-22.21±1.28	63.54±4.42
F6	84.06±9.36	7.12±2.80	-24.64±0.53	66.98±3.83
F7	79.11±3.96	3.68±0.69	-30.04±0.72	74.04±2.25
F8	83.47±2.65	3.09±1.32	-28.84±0.47	75.33±0.89
F9	72.33±2.03	4.24±0.41	-27.32±0.34	71.05±0.93

Evaluation of liposomes

Antigen entrapment efficiency (% EE)

The proportion of entrapped antigen was obtained by ultracentrifugating 1 ml of the liposomal suspension at 15,000 rpm for 1 hr using a cooling centrifuge at 4^oC (Remi C-24, Mumbai, India). The liposomes were separated from the supernatant and were washed twice, each time with 1 ml distilled water, and recentrifuged again for 1 hr. The amount of entrapped antigen was determined by lysis of the separated vesicles with Triton X-100. 0.2 ml of the liposomal formulation was taken and about 0.2 ml of 1% Triton X-100 was placed into it. This was kept for 5 minutes for disrupting the vesicles. Then vesicle was centrifuged for 5 minutes at 2000 rpm. The supernatant was collected and used for the quantization of antigen entrapped by BCA method using U. V. spectrophotometer (UV 1700 Pharm Spec, Shimadzu, Japan) at 562 nm.

The proportion of encapsulated pilocarpine hydrochloride was obtained by A 100 ll sample of niosomes was mixed with 1 ml of isopropanol; the volume was completed to 10 ml with phosphate buffered saline and covered with parafilm to prevent evaporation. The concentration of the drug was determined by UV spectrophotometer at 215 nm. Study was done in triplicate and % drug entrapment efficiency can be calculated by using following formula:

$$\% EE = \frac{\text{Actual drug content}}{\text{Theoretical drug content}} \times 100$$

Table 5.1: **Optimization of PC: PE: Cholesterol ratio wrt size and % entrapment**

S.No.	Code	PC:PE:Chol (molar ratio)	Average size (µm)	% antigen entrapped
1	CL1	8:0:2	1.55±0.19	44.23±0.8
2	CL2	7.2:0.5:2	1.75±1.04	52.89±0.67
3	CL3*	7.0:1.0:2	2.52±0.54	60.10±1.45
5	CL4	6.6:1.4:2	1.98±0.38	54.28±0.64
6	CL5	6.2:1.8:2	1.59±0.49	49.39±0.52

CHARACTERIZATION OF LIPOSOMES

Vesicle shape

The prepared liposomal formulations were characterized for their shape using transmission electron microscopy (TEM). (Guo et al.; 2000).

Protocol 4.6: Transmission Electron Microscopy (TEM)

Equipment and Reagent:

Electron Microscope(JEM-200 CX, JEOL, Tokyo, Japan) Liposome formulation

1% phosphotungstic acid carbon coated grid

Method:

1. A drop of liposome formulation was placed on to the surface of butter paper.
2. Carbon coated grid was then placed over the drop of 1% Phosphotungstic acid and again excess of PTA was removed by whatman filter paper.
3. The grid was observed under the electron microscope & photo taken.

Vesicle size and Size Distribution

The size and size distribution of vesicles was determined by particle size analyzer (Cilas, 1064 L, France).

Vesicle Count

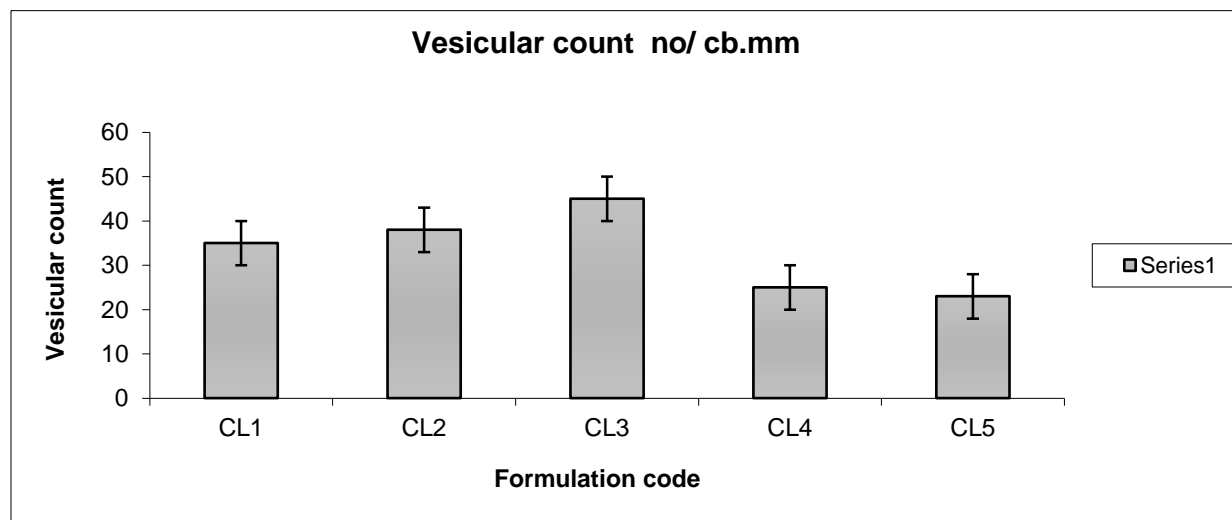
Liposomal preparations were diluted 10 times with 0.9% w/v sodium chloride solution and no. of vesicle /mm³ were counted by optical microscopic method using hemocytometer. The liposome in 80 small squares counted and total no of vesicle/mm³ were calculated using following formula.

$$\text{Total no. of vesicles/mm}^3 = \frac{\text{Total no. of vesicles} \times 4000 \times \text{Dilution factor}}{\text{Total no. of squares counted}}$$

The observed values were recorded in table 5.3 and presented graphically in figure 5.3.

Table 5.3: Vesicle count of PC:PE:CH liposome

S. No.	Formulation code	Vesicle count/ mm ³
1.	CL1	35±2
2.	CL2	38±3
3.	CL3	45±4
4.	CL4	25±2
5.	CL5	23±3

Figure 5.3: Vesicle count of PC:PE:CH liposome

Percent of antigen entrapped in liposomes was determined by method described by Fry et al. (1978) with slight modification in purification of liposomes was done by using micro centrifuge column of sephadex G-50 and then entrapment efficiency was measured by rupturing the vesicles with triton X -100 (1.0%).

Preparation of Minicolumn

Column of sephadex G-50 gel was prepared as described by Fry et al. (1978) by slight modification.

Methods:

1. Sephadex G-50 was accurately weighted 1g and swollen in 10ml of 0.9% NaCl solution for 12 hrs at room temperature with occasional shaking.
2. The barrel was plugged with whatman filter paper pad.
3. The prepared gel was filled to the top in the barrel of 1ml disposable syringe.
4. The barrel was then placed in the centrifuge tube.
5. The tubes were centrifuged at 3000rpm for 3minutes to remove excess saline.

Purification of liposome**Method:**

1. Prepared minicolumn was taken and then 0.2ml of liposomal preparation was placed on the sephadex bed.
2. The minicolumn was then spine for 3 minutes at 1000 rpm to expel the liposomal material from the column into the test tube.
3. Non-Encapsulated solute from the column was removed by washing with buffer and eluted by centrifugation at 3000 rpm.

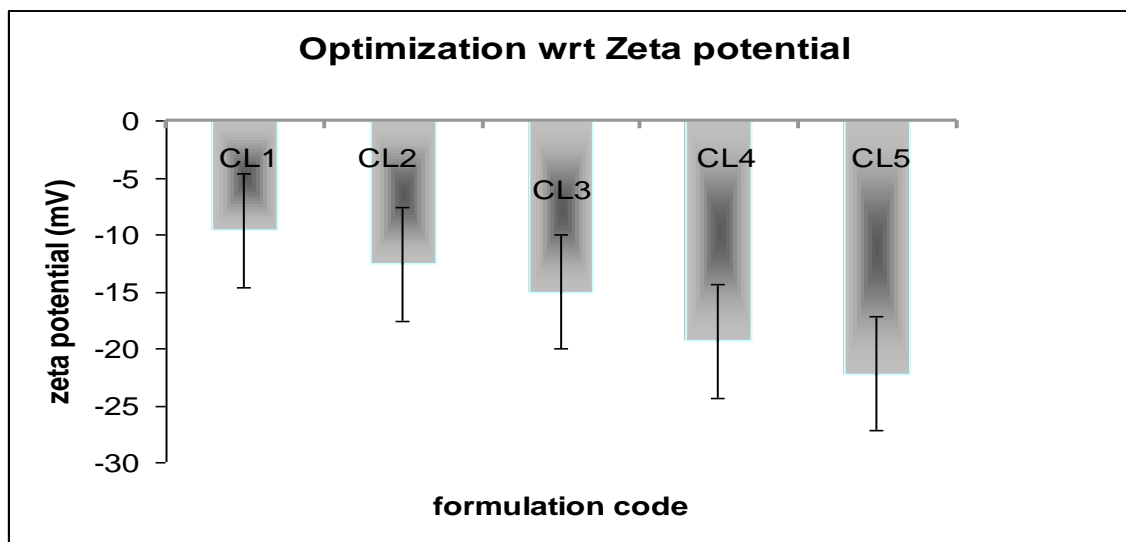
Surface Properties of Liposomes

The change in surface property of the liposomes were evaluated by measuring the zeta potential of the prepared liposomal formulation with a zeta meter (Zetasizer, Malvern. UK) (Tatiana et al.;2002). The observed values were presented in table-5.4 and graphically in figure 5.4.

Table 5.4: Zeta potential of different formulation of CL liposome

S. No.	Formulation code	Zeta potential(mV)
1.	CL1	-9.64±0.53
2.	CL2	-12.54±0.72
3.	CL3*	-15.04±0.47
4.	CL4	-19.32±0.34
5.	CL5	-22.21±1.28

Figure 5.4: Zeta potential of Optimized formulations



RESULTS & DISCUSSION

BSA loaded liposomes of Soya PC:PE:CH were prepared by reverse phase evaporation method. Reverse phase evaporation was selected because it was reported that higher encapsulation efficiency of macromolecule (protein and peptide) can be achieved by this method, moreover cast film method is associated with relatively mild processing conditions (no exposure to organic solvent, sonication etc.) and help in the maintenance of three-dimensional structural integrity of protein. The result revealed that the liposomes can be prepared by any of the method described for the preparation of liposome i.e. entrapment efficiency was found to be more in the case of vesicle prepared by reverse phase evaporation method.

The Soya PC:PE:CH liposomes were prepared with 7:1:2, PC:PE and cholesterol molar ratio and optimized. Entrapment efficiency of conventional Soya PC:PE:CH liposomes prepared by reduced phase evaporation was found to be $60.10 \pm 1.45\%$. The Soya PC:PE:CH liposomes prepared by reverse phase evaporation method were selected for further study and optimized on the basis of particle size, particle count, entrapment efficiency and *in-vitro* release.

Transmission electron microscopy and light microscopy were used to study shape and lamellarity of the vesicles which revealed unilamellar and spherical shape of the conventional Soya PC:CH liposomes. The particle size of liposomes decrease with increasing sonication time the particle size was measured to be $2.52 \pm 0.12 \mu\text{m}$ for formulation CL-1, $2.02 \pm 0.21 \mu\text{m}$ for formulation CL-2, $0.89 \pm 0.29 \mu\text{m}$ for formulation CL-3, $0.56 \pm 0.45 \mu\text{m}$ for formulation CL-4, $0.33 \pm 0.15 \mu\text{m}$ for formulation CL-5.

Zeta potential of optimized Soya PC:PE:CH liposomes was found to be $-15.04 \pm 0.45 \text{mV}$ which is significantly low indicates that PE might have responsible for the lowering of zeta potential.

***In-Vitro* Antigen release**

The *in-vitro* antigen release profile of entrapped antigen from selected formulations were studied using dialysis tube (Mohamed et. al. 2006), treatment of cellophane membrane was done by adopting the protocol 4.7 and release profile was determined by following the protocol 4.8.

Various liposomal formulations were first centrifuged through mini-column to remove untrapped antigen. 1.0mL of pure liposomal suspension, free of any untrapped antigen was taken into a dialysis tube and placed in a beaker containing 20.0 ml of PBS pH 7.4. The beaker was placed over a magnetic stirrer and the temperature of the assembly was maintained at $37\pm 1^{\circ}\text{C}$ throughout the study. Samples were withdrawn at predetermined time intervals and replaced with the same volume of PBS pH 7.4. The withdrawn samples were diluted suitably and absorbances of the solution were measured at 280nm against PBS pH 7.4 as blank. The antigen content was calculated and recorded in table 7.1 and presented graphically in figure 7.1.

Protocol 4.7: Treatment of cellophane membrane**Equipment and Reagent:**

Cellophane membrane	0.2% v/v sulphuric acid
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Magnetic stirrer with hot plate	PBS(pH 7.4)
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0.3% w/v solution of sodium sulphite

Method:

1. The cellophane membrane was kept in beaker with water and stirred for 12 hrs to remove glycerin based content.
2. The sulphur base content was removed by treating the membrane with 0.3% w/v sodium sulphite at 70°C for 20 minutes.
3. Membrane was again treated with 0.2% v/v of sulphuric acid. This membrane was kept in PBS (pH 7.4) until used for release profile study.

Protocol 4.8: *In- vitro* % release

Equipment and Reagent:

Treated cellophane membrane Beaker 100ml

1ml liposomal formulation 50 ml PBS

Magnetic stirrer with hot plate

Method:

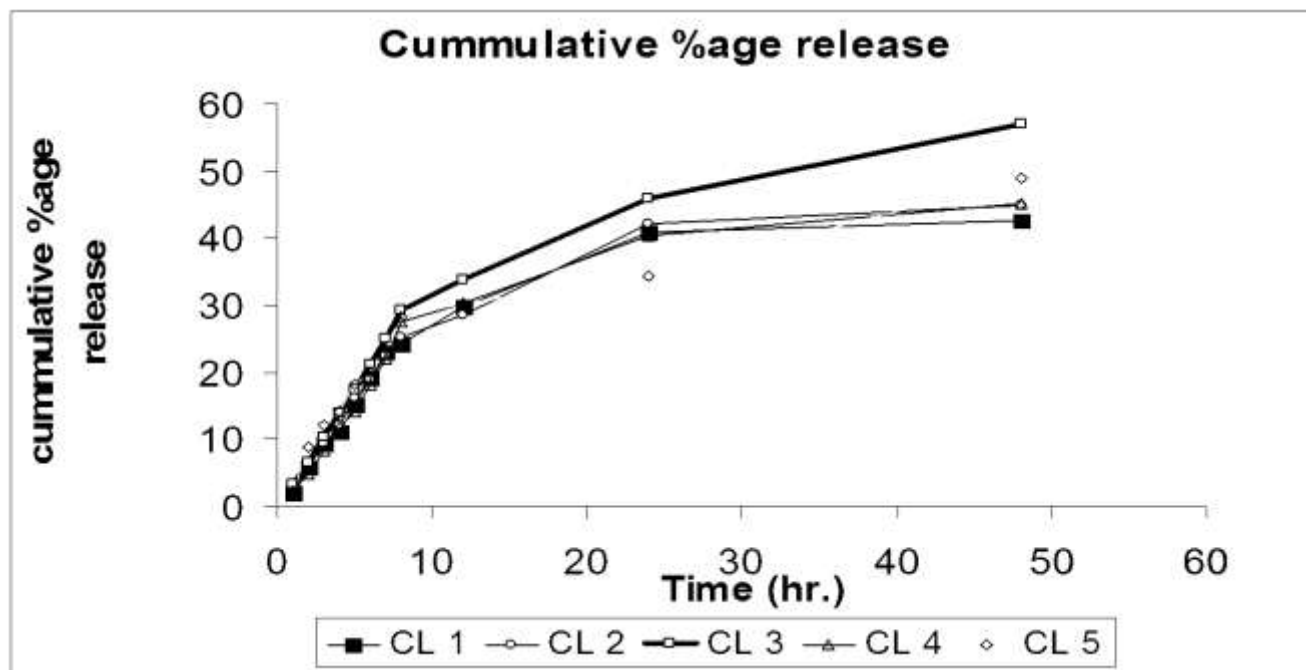
1. The cellophane membrane was placed on two side open hollow tube with the thread.
2. Purified liposomal formulation (1ml) placed in that tube.
3. The tube was placed in a beaker containing 50 ml PBS.
4. The solution containing the tube was slowly stirred by a magnetic bead using magnetic stirrer with hot plate and temperature was kept constant at $37\pm 1^{\circ}\text{C}$ throughout the study.
5. Samples were withdrawn at predetermined time interval and replaced with the same volume of PBS buffer.
6. Samples were analyzed for antigen content by diluting suitably and absorbance of the solution was taken at λ_{max} 280 nm against blank.

Table 7.1: *In- vitro* release of the antigen

S.N.	Time (hrs)	CL1	CL2	CL3*	CL4	CL5
1	1.0	2.12±0.05	3.0±0.26	3.32±0.06	2.22±0.26	2.25±0.55
2	2.0	5.7±0.3	6.567±0.2	6.67±0.2	4.67±0.36	8.88±0.52
3	3.0	9.32±0.11	09.32±0.13	10.22±0.13	8.22±0.13	12.22±0.53
4	4.0	11.21±0.22	12.22±0.31	13.82±0.21	11.82±0.21	14.23±0.51
5	5.0	15.23±0.18	16.22±0.18	17.52±0.18	14.2±0.18	18.25±0.38
6	6.0	19.22±0.63	19.02±0.23	21.22±0.23	18.22±0.23	20.00±0.13
7	7.0	22.98±0.41	22.32±0.11	25.02±0.31	22.02±0.21	23.52±0.31
8	8.0	24.32±0.33	25.32±0.20	29.32±0.30	27.542±0.10	28.2±0.30
9	12.0	29.84±0.19	28.54±0.19	33.84±0.19	30.24±0.29	30.21±0.25
10	24.0	40.84±0.11	42.2±0.23	45.84±0.23	40.23±0.52	34.23±0.22

11	48.0	42.58±0.23	44.84±0.19	56.90±0.21	45.23±.23	48.8±0.33
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Fig 7.1: *In-vitro* release of antigen.



RESULTS & DISCUSSION

In-vitro release profile of prepared formulation was determined by method described by Mohammed *et.al*. The study was carried out for 48hrs and withdrawn samples were analyzed spectrophotometrically at 280nm. Cumulative percentage release obtained after 48h for formulation CL3 was 56.90±0.21%. The formulation CL3 prepared by reverse phase evaporation was evaluated for entrapment of antigen by rupturing the vesicles by triton X-100 (1.0%). Entrapment efficiency of antigen was found 60.10±1.45%.

Stability Study

Stability is defined as the extent to which a product retains the same properties and characteristics within specified limits possessed at the time of its manufacture in specific container or closure system.

The prepared formulations were tested for stability on storing them at 4±1°C and at 25±1°C (pH-7.4) for 30 days. The formulations were evaluated for the following parameter.

1. Vesicle size and shape
2. Residual antigen content
3. Antigen integrity

EFFECT OF STORAGE ON VESICLE SIZE

Size of the vesicles was determined initially and after 30 days of storage by using particle size analyzer (CILAS, 1064 L, France). Effect of storage on vesicle size has been shown in table 6.1 and graphically shown in figure 6.1.

EFFECT OF STORAGE ON RESIDUAL ANTIGEN CONTENT

Selected formulation was stored for 30 Days and evaluated for residual antigen content. Samples were taken at appropriate time intervals and estimated observations are recorded in table 6.2 and depicted graphically in figure 6.2.

ANTIGEN INTEGRITY

The structural integrity of antigen after formulation and on storage at $4 \pm 1^\circ\text{C}$ and at $25 \pm 1^\circ\text{C}$ was assessed after 30 days by performing the SDS –PAGE for the protein adopting the protocol 4.3. Results of the study are shown in photograph (fig; 9.5C).

Table 6.1: Effect of storage on vesicle size

S. No.	Formulation code	Initial size(μm)	Final size(μm) At $4\pm 1^\circ\text{C}$	Final size(μm) At $25\pm 1^\circ\text{C}$
1	CL3	2.52 ± 0.54	2.96 ± 0.34	3.2 ± 0.5

Figure 6.1: Effect of storage on vesicle size

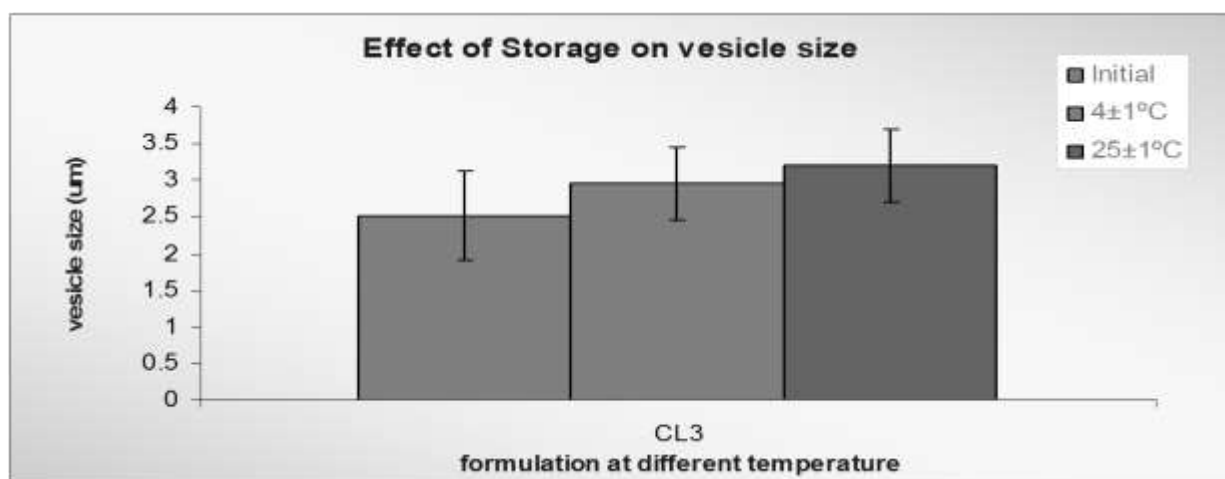
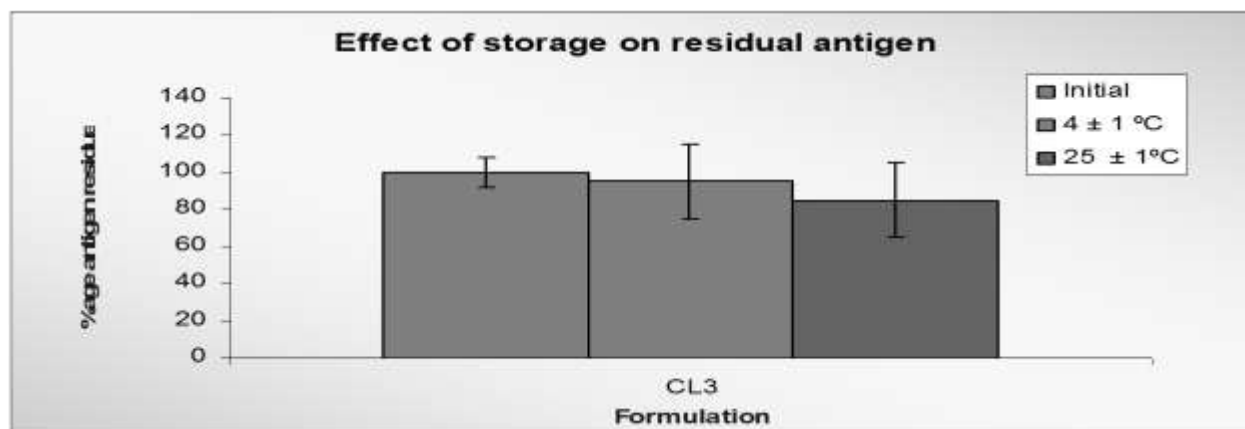


Table 6.2: Effect of storage on residual antigen content

S. No.	Formulation code	Initial	Residual antigen At $4 \pm 1^\circ\text{C}$	Residual antigen At $25 \pm 1^\circ\text{C}$
1	CL3	100%	$95.0 \pm 2.0\%$	$84.8 \pm 2.5\%$

Figure 6.2: Effect of storage on residual antigen content

RESULTS & DISCUSSION

Lipid vesicles are self assembles of amphiphiles into closed bilayer structure. Hydrated bilayer vesicles, however are not considered as thermodynamically stable and thought to represent a metastable state in the vesicle possess an excess of energy. Bilayer phospholipids can undergo chemical degradation such as oxidation and hydrolysis. Due to these changes vesicular systems maintained in aqueous dispersion might aggregate and hence encapsulated bioactive material may tend to leak out from the bilayered structure during storage. A stable formulation must exhibit constant particle size and a constant level of entrapped antigen throughout the storage period. In the present study the storage stability of the vesicles was determined by measuring the vesicle size, residual antigen content and antigen integrity before and after 30 Days at $4 \pm 1^\circ\text{C}$ and $25 \pm 1^\circ\text{C}$. In Soya PC:PE:CH liposomes only a slight increase in size on storage at $25 \pm 1^\circ\text{C}$ and insignificant change in size on storage at temperature $4 \pm 1^\circ\text{C}$ occur, indicate that Soya PC:PE:CH liposome are more stable than conventional Soya PC:CH liposomes. Percent of residual antigen remain in liposomes by assuming the initial content to be 100% ,in Soya PC:PE:CH liposomes only 14-15% antigen was lost at temperature $25 \pm 1^\circ\text{C}$ and 5-6% antigen was lost on storage at $4 \pm 1^\circ\text{C}$.

Antigen integrity was evaluated by performing the SDS -PAGE of the liposome formulations. (Optimized CL3 formulation stored at $4 \pm 1^\circ\text{C}$) after 30 Days. Antigens were found to be intact in the formulation stored at $4 \pm 1^\circ\text{C}$ after 30 days. There was no effect of storage on structural integrity of the antigen (fig; 9.5B and fig; 9.5C).

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

This protocol was approved by the Institutional Animal Ethical committee of CSIR-CDRI which implements the national guidelines of CPCSEA for use and handling of animals.

HUMAN AND ANIMAL RIGHTS

This article does not contain any studies with human subjects performed by any of the authors; all institutional and national guidelines for the care and use of laboratory animals were followed.

CONFLICT OF INTEREST

The authors report no conflicts of interest.

DATA AVAILABILITY STATEMENT

Not applicable

INFORMED CONSENT STATEMENT

Not applicable

AUTHOR CONTRIBUTIONS

The corresponding author designed and performed the experiments. The co-authors supervised the findings of this work. All authors discussed the results and contributed to the final manuscript.

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