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Research Article

LIPOSOMAL DELIVERY OF IMMUNOMODULATORY ANTIGEN OF *B. MALAYI:* ISOLATION, PREPARATION, CHARACTERIZATION, AND IMMUNE RESPONSES ASSESSMENT

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ABSTRACT

Objective: The present study was aimed on developing and characterizing liposomal delivery system loaded with antigen of filaria parasite *Brugia malayi* extracted protein for assessment of humoral immune responses of antigen.

Methods: Liposomes were prepared by reverse-phase evaporation method with slight modification using molar ratio of Soya PC: PE:Cholesterol in different molar concentrations.

Results: The levels of F6 specific immunoglobulin (Ig) G1, IgG2a and IgG2b antibodies were found to be elevated in immunized animals over non-immunized controls. Analysis of IgG-subclasses revealed that all the subclasses at (1:25dilution) increased several folds over the controls with IgG1 showing the greatest increase (25.0-fold) followed by IgG2b (3.0-fold). Antibodies titers showed the many fold increment of titers on liposomized antigen groups (Gr.I; without booster dose and Gr.IV; with booster dose). IgG showed about 2.2 fold increment in Gr.IV than control group (Gr.V). IgG1 after booster dose showed about 25-fold increment followed by IgG2b than IgG2a.

Conclusion: These results suggest that the liposomal antigen delivery system shows 25-fold IG-G responses in comparison to plain administrated antigen.

Keywords: Ig-G, BmAFII, *Brugia malayi*, Liposomes, Humoral immunity.

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INTRODUCTION

Lymphatic filariasis (LF) – 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 [1]. Approximately 125 million already have been infected with LF, and over 40 million [2] are seriously incapacitated and disfigured by the disease.

In recent years, identification of several filarial antigens/proteins or molecules raised hopes for developing vaccines [3-8] against LF.

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.

Studies conducted in laboratory revealed that of the two major Sephadex G-200 eluted fractions of *Brugia 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. Further, to narrow down to molecular entities that have cytokine release stimulating potential, *B. malayi* adult worm extract was fractionated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and the resolved fractions were blotted onto NCP and screened for the cytokine release potential. 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.

Liposomes have been used in drug delivery for many years. Liposomes, discovered in 1965 by Bangham *et al*., are spherical colloidal particles containing an aqueous core surrounded by phospholipid bilayer which replicates cell membrane. The drug carrying capacity, release rate, and deposition of liposomes are 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 used are the lecithins (phosphatidylcholines), phosphatidylethanolamines (PE), sphingomyelins, phosphatidylserines, phosphatidylglycerols (PG), and phosphatidylinositols (PI) [9].

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, 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, and preparation process is simple and easy to scale up for chemical studies and eventual manufacturing. 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 access immune-adjuvancity of prepared dosage form in antigen dose reduction by using different immunization protocols.

METHODS

PC: PE:CH were generous gift from Lipoid, Germany. SDS-PAGE reagents were Sigma, USA. All other chemicals and reagents are available at in-house facility of CDRI, Lucknow.

Antigen isolation

Parasite

For laboratory experimental purpose, *B. Malayi*, having many of the biological aspects like *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 Fig. 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. *M. coucha* model is found amenable to perform chemotherapeutic and immunobiological investigations in experimental filariasis [10]. Keeping in view their similarity of immune responses to human, this animal is used as model for experimental purpose another rodent, the Mongolian gerbil (*Meriones unguiculatus*, family Gerbillinae) as shown in Fig. 1 has proven to be an excellent permissive rodent model for the study of LF using *Brugia pahangi* or *B. malayi*. The animal (called "jird") is used for the propagation of *B. malayi* parasites [11]. 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.

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 h 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 h and these were provided with feed containing dog box and yeast powder. The larvae usually took about 8–10 days to become pupae, which ultimately developed into adult mosquitoes within 48 h.

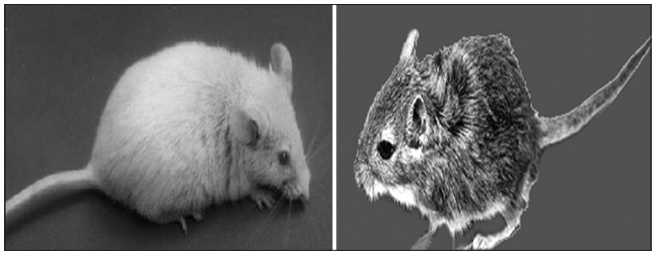

Fig. 1: Experimental filarial models – *Mastomys coucha* and *Meriones unguiculatus*

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 h were allowed to feed on the donors, which was kept inside the mosquito cage in a wire netting immobilized cage. After 1 h 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 L_3.

Isolation of L3 from mosquitoesOn 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 Baermann 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 L_3 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-week-old male *M. coucha* were inoculated with active and motile $L_{3(}$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 L_3 were inoculated intraperitoneally. The larvae develop into adult worms in about 3 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 s each at 10-micron amplitude with intermittent gap of 1 min. The total homogenate was mixed with equal amount of 2× sample buffer for sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS PAGE) (0.125 M Tris-HCl, pH 6.8; 4% SDS, 5% β-mercaptoethanol, 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.

SDS-PAGE

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. To isolate dominating fractions which have been identified to be stimulators of pro- and mixed pro- and anti-inflammatory cytokine release [12], extract of adult worms was used. The extract was resolved in 10% gels [13]. 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.

Electro-elution of proteins from gelProteins 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 2–4 h 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 2000 g 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 segments of unequal size. It must be protected from light and moisture and store at temperature between 2°C and 25°C (Table 1).

B. 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, elongation factor 2, and 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) [14] with slight modification using different molar ratio of Soya PC: PE:Cholesterol. PC: PE:CH was dissolved in 5 mL of diethyl ether (Table 2). To the above solution, 3 mL of PBS pH 4.2 was added. The mixture was sonicated for 1.0 min at 4°C. The mixture was kept in a rotary vacuum evaporator; the organic solvent was removed under vacuum (260–400 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 3).

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 h using a cooling centrifuge at 4°C (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 h. 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 min for disrupting the vesicles. Then, vesicle was centrifuged for 5 min 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.

Characterization of liposomes

Vesicle shape

The prepared liposomal formulations were characterized for their shape using transmission electron microscopy (TEM).

Vesicle size and size distribution

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

Table 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-1β (+++)
F6 (54.3–67.8)*	IL-1β (+++), IL-6 (+++), TNF- α (+)
F10 (38.44–41.84)	IL-1β (++)
F14 (17.0–22.5)	IL-10 (+)

+, ++ and +++ indicate ascending grade of predominance. *selected for present study

Table 2: 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: Optimization of PC: PE: Cholesterol ratio with respect to 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

Vesicle count

Liposomal preparations were diluted 10 times with 0.9% w/v sodium chloride solution and number of vesicle/mm^3 were counted by optical microscopic method using hemocytometer. The liposome in 80 small squares counted and total number of vesicle/mm^3 were calculated using following formula.

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

The observed values were recorded in Table 4 and presented graphically in Fig. 2.

Percent entrapment

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 using microcentrifuge 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 10 mL of 0.9% NaCl solution for 12 h 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 3000 rpm for 3 min to remove excess saline.

Purification of liposome

Method

1. Prepared minicolumn was taken and then 0.2 mL of liposomal preparation was placed on the Sephadex bed.
2. The minicolumn was then spine for 3 min 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) [15]. The observed values are presented in Table 5 and graphically in Fig. 3.

***In-vivo* studies**

The work was carried out with an aim to develop delivery system that can deliver parenterally administered antigen in its immunologically active form to their target site to elicit immune response. *In vivo* experiments were performed to evaluate the therapeutic and prophylactic effectiveness of the antigen as well as drug delivery system.

Table 4: 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

Table 5: 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

Table 6: Immunization protocol

S. No.	Group	Formulation administered	No. of animals
1.	I	Liposomized antigen (Single dose)	5
2.	II	Plain antigen (Single dose)	5
3.	III	Plain antigen (Double dose)	5
4.	IV	Liposomized antigen (Double dose)	5
5.	V	P.B.S.	5

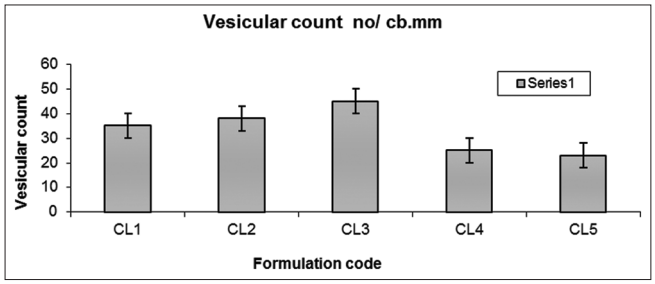

Fig. 2: Vesicle count of PC:PE:CH liposome

Immunization of animals

The work was carried out with an aim to develop delivery system that can deliver parenterally administered antigen in its immunologically active form to their target site to elicit immune response and the justification for the use of animals is that the real-time immune responses are to be assessed to measure the efficacy of antigen and delivery system in the way to develop an effective vaccine candidate against the disease. Immunization of Animals, Swiss mice (Male, 4–6 weeks old and 20–25g body weight) were taken and the anesthesia is used as Lidocaine Hydrochloride 0.5% (7 mg/kg of body weight) sub-cutaneously (s.c.). This protocol was approved by the Institutional Animal Ethical committee of CSIR-CDRI which implements the national guidelines of Committee for the Purpose of Control and Supervision of Experiments on Animals, Department of Animal Husbandry, Dairying and Fisheries, Govt. of India (Registration Number 34/GO/ReBiBt-S/Re-L/99 CPCSEA). The facility is GLP compliant for conducting toxicity and safety pharmacology studies under the regulatory ambit of NGCMA, Department of Science and Technology, Government of India. The facility strictly adheres to the principle of 4 Rs (reduce, refine, replacement, rehabilitation) in animal experimentation. The *in vivo* experimentations, animal health, and well-being are monitored by trained technicians, experienced scientists, and veterinarians. The studies were carried out as per the guidelines of Council for Purpose of Control and Supervision of Experiment on Animal, Ministry of Social Justice and Empowerment, Government of India. This study included 5 groups of animals, each group comprising of 5 animals (total 25 animals). The dose administered 250.00 µL of formulation administered subcutaneously (s.c.). The protocol followed as given in Table 6:

Sample collection

The pre-immune samples of serum were collected on day 0 before immunization and post-immunization samples collected on day 7, 14, 21, and 28th. Blood from each mouse was taken in an Eppendorf tube through the retro orbital plexus with the help of glass micro capillaries, allowed to clot, and then, centrifuged to separate serum. All the collected samples were stored at −20°C until utilized. On day 28th, all the animals were sacrificed and spleen and peritoneal fluid were collected for cell mediate immune response and cytokines determination. The peritoneal macrophages were isolated aseptically for Nitric-Oxide determination [16-21].

Humoral immune (HI) responses measurements:

Determination of Immunoglobulin (Ig)G antibody and subtypes

Filaria-specific IgG and its subtypes were detected in sera of animals. Briefly, ELISA strips (Greiner Bione) were coated with the fraction F6 (0.1µg protein/mL) prepared in carbonate buffer (0.06M; pH 9.6). Optimally diluted sera were used (IgG 1:100 and IgG subclasses 1:25) and probed with HRP-conjugated rabbit anti-mouse-IgG and its subtypes (Sigma Chem. Co, USA) at 1:1000 dilution. Ortho phenylenediamine was used as substrate and absorbance was read at 492 nm in an ELISA reader (PowerWaveX, BioTek, USA). Results are shown in Table 7a-e and graphically presented in Fig. 4a-e.

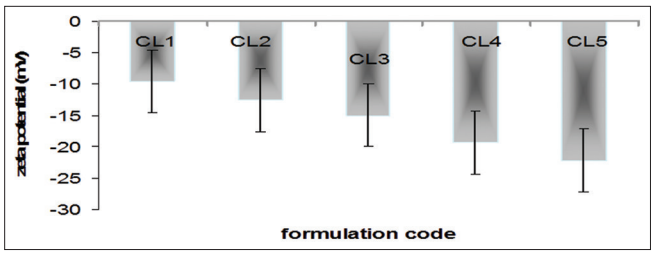

Fig. 3: Zeta potential of optimized formulations

Table 7a: IgG titer of different groups after immunization (Mean±SD) (n=5)

Groups/time-point (days)	Formulation group I		Formulation group II		Formulation group III		Formulation group IV		Formulation group V	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
7th	0.58	0.02	1.03	0.05	0.66	0.03	0.09	0.01	0.15	0.06
14th	0.88	0.06	0.74	0.04	0.64	0.04	0.15	0.01	0.14	0.03
21th	0.73	0.02	0.66	0.02	0.78	0.12	2.05	0.08	0.17	0.01
28th	1.09	0.64	1.04	0.05	1.13	0.04	2.05	0.07	0.03	0.02

Table 7b: IgG1 titer of different groups after immunization (Mean±SD) (n=5)

Groups/time-point (days)	Formulation group I		Formulation group II		Formulation group III		Formulation group IV		Formulation group V	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
7th	0.15	0.01	0.05	0.01	0.12	0.01	0.31	0.02	0.02	0.01
14th	0.05	0.02	0.02	0.01	0.04	0.01	0.07	0.01	0.02	0.02
21th	0.06	0.01	0.02	0.01	0.05	0.01	2.59	0.09	0.01	0.05
28th	0.07	0.01	0.04	0.02	0.09	0.01	2.42	0.05	0.03	0.04

Table 7c: IgG2a titer of different groups after immunization (Mean±SD) (n=5)

Groups/time-point (days)	Formulation group I		Formulation group II		Formulation group III		Formulation group IV		Formulation group V	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
7th	0.17	0.06	0.20	0.06	0.23	0.02	0.21	0.04	0.40	0.38
14th	0.13	0.11	0.17	0.02	0.11	0.05	0.12	0.01	0.08	0.01
21th	0.06	0.05	0.13	0.02	0.14	0.05	0.21	0.07	0.07	0.04
28th	0.21	0.05	0.16	0.01	0.14	0.01	0.17	0.03	0.50	0.33

Table 7d: IgG2b titer of different groups after immunization (Mean±SD) (n=5)

Groups/time-point (days)	Formulation group I		Formulation group II		Formulation group III		Formulation group IV		Formulation group V	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
7th	0.49	0.05	0.18	0.02	0.48	0.06	0.65	0.05	0.98	0.12
14th	0.44	0.07	0.35	0.08	0.47	0.05	0.20	0.02	0.91	0.12
21th	0.33	0.06	0.35	0.08	0.55	0.02	1.22	0.13	0.41	0.54
28th	0.57	0.27	0.68	0.01	0.91	0.11	0.93	0.06	0.30	0.42

Table 7e: IgG3 titer of different groups after immunization (Mean±SD) (n=5)

Groups/time-point (days)	Formulation group I		Formulation group II		Formulation group III		Formulation group IV		Formulation group V	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
7th	0.11	0.14	0.39	0.34	0.06	0.01	0.10	0.01	0.32	0.01
14th	0.07	0.03	0.06	0.01	0.22	0.01	0.05	0.01	0.26	0.02
21th	0.08	0.04	0.05	0.01	0.19	0.01	1.11	0.04	0.03	0.03
28th	0.21	0.15	0.29	0.01	0.09	0.01	0.66	0.01	0.25	0.16

RESULTS AND DISCUSSION

BSA-loaded liposomes of Soya PC: PE:CH were prepared by REV method. REV 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 REV 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±1.45%. The Soya PC: PE:CH liposomes prepared by REV method were selected for further study for immunomodulatory effect through *in vivo* and animal studies. TEM 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 decreases with increasing sonication time the particle size was measured to be 2.52±0.12 μm for formulation CL-1, 2.02±0.21 μm for formulation CL-2, 0.89±0.29 μm for formulation CL-3, 0.56±0.45 μm for formulation CL-4, 0.33±0.15 μm for formulation CL-5. Zeta potential

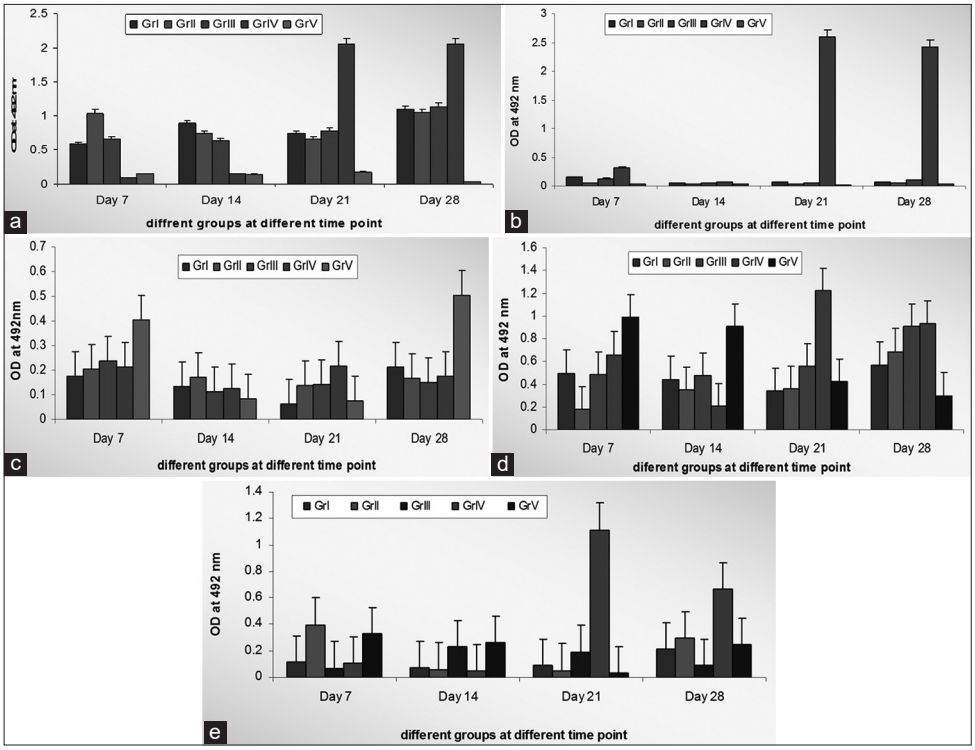

Fig. 4: (a) IgG titer of different groups after immunization (Mean±SD) (n=5). (b) IgG1 titer of different groups after immunization (Mean±SD) (n=5). (c) IgG2a titer of different groups of after immunization (Mean±SD) (n=5). (d) IgG2b titer of different groups after immunization (Mean±SD) (n=5). (e) IgG3 titer of different groups after immunization. (Mean±SD) (n=5)

of optimized Soya PC: PE:CH liposomes was found to be −15.04±0.45 mV which is significantly low indicates that PE might have responsible for the lowering of zeta potential.

The levels of F6 specific IgG1, IgG2a and IgG2b antibodies were found to be elevated in immunized animals over non-immunized controls. Analysis of IgG-subclasses revealed that all the subclasses at (1:25dilution) increased several folds over the controls with IgG1 showing the greatest increase (25.0-fold) followed by IgG2b (3.0fold). Antibodies titers showed the many fold increment of titers on liposomized antigen groups (Gr.I; without booster dose and Gr.IV; with booster dose). IgG showed about 2.2 fold increment in Gr. IV than control group (Gr.V). IgG1 after booster dose showed about 25-fold increment followed by IgG2b than IgG2a.

CONCLUSION

These results suggest that the liposomal antigen delivery system shows 25-fold IG-G responses in comparison to plain administrated antigen.

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.

DATA AVAILABILITY STATEMENT

All the related data are with author (Neeraj Jain) and can be represented whenever asked.

INFORMED CONSENT STATEMENT

Not applicable.

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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.

CONFLICT OF INTEREST

The authors report no conflicts of interest.

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