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Polysaccharide coated liposomes for oral immunization — development and characterization

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Abstract

Polysaccharide coated liposomes were prepared, characterized and evaluated for their potential use in oral immunization. Liposomes were prepared by reverse phase evaporation method. Bovine serum albumin (BSA) was chosen as the model antigen. Pulluan, a naturally occurring polysaccharide produced by a yeast like fungus, was chemically modified into its palmitoyl derivative (*O*-palmitoylpullulan; OPP) and was used for coating of the liposomes. The synthesized OPP was characterized by IR and NMR spectroscopy. The liposomes prepared were characterized for their size, shape, surface charge, encapsulation efficiency and stability in simulated gastric fluid. The immune stimulating activity was studied by measuring the serum IgA and IgG following oral administration of the prepared polysaccharide coated liposomes. Similarly, other formulations were studied and the results were compared. BSA loaded liposomes coated with OPP and plain polysaccharide could produce better IgG and IgA titre levels as compared to plain alum adsorbed BSA. The plain liposomes containing BSA could however produce significantly higher IgG and IgA levels as compared to equivalent BSA–alum based oral immunization. The results indicate that chemically modified polysaccharide coated liposomes can be used as a potential adjuvants for effective oral immunization.

Keywords: Liposomes; Oral immunization; *O*-palmitoylpullulan; Polysaccharide

1. Introduction

Immunization is the most efficient and cost-effective means for the prevention of various diseases. The mucosal surface of the human body is a major site of entry for pathogens. However, no significant means of immunization have been brought about to prevent this. Historically immunization has relied on the induction of humoral immunity by parenteral administration of vaccines. Moreover, antibodies induced in this manner, do not always reach the mucosal surfaces which is the predominant site for most of the infectious pathogens.

Among the antibodies induced in this manner, it is not always necessarily reach the mucosal surfaces which is the predominant site for most infectious pathogens. Among the antibodies produced in the body, antibodies produced by hu-

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Table 1
Various formulation codes and encapsulation efficiency and size of liposomes

Formulation code	Molar lipid ratio (PC:CH:PE)	Encapsulation efficiency (%)	Size (μm)
P1	9:1:0	37.5 ± 0.9	4.3–5.4
P2	7:2:1	40.3 ± 1.8	2.5–3.35
P3	5:4:1	35.6 ± 1.2	5.9–8.5

moral immune responses at the mucosal surfaces are of importance in fighting against pathogens at the mucosal surface. One such antibody is the secretory IgA (sIgA). Secretory IgA is the predominant antibody isotype present at the mucosal sites which prevents the attachment of bacteria and viruses to the mucosa and, thereby, prevents any possible damage to the host.

Mucosal immunity provides the first line of immunological defence. However, to induce antibodies at the mucosal surfaces administration of antigen directly to the mucosal site is required. Oral immunization offers the safest and most convenient way to induce mucosal immunity (Mestecky, 1987). Orally administered antigens are taken up through the specialized epithelial cells called M cells, overlaying Peyer's patches which are the major component of the gut associated mucosal immune system. The picked up antigens are transported into the regional lymphoid tissues, processed and stimulate antigen specific B lymphocytes in the germinal centres of follicles located beneath domes.

Oral administration of vaccines generally requires large doses of antigen due to insufficient stimulation of gut associated lymphoid tissue (GALT). In addition oral administration of antigens must overcome several challenges. To overcome these disadvantages encapsulation of antigens and delivering it in a safer way in order to stimulate mucosal immunity has been proposed over the years.

Among other delivery systems for oral immunization liposomes have a number of potential advantages (Alving, 1987; Childers and Michalek, 1994). Other delivery systems studied include microspheres (Moloveanu et al., 1993), alginate microparticles (Bowersock et al., 1996), polymerized liposomes(Chen et al., 1996), hydrogels (Bowersock et al., 1994), niosomes (Brewer and Alexander, 1992), Novasomes® (Gupta et al., 1996), etc.

The susceptibility of conventional liposomes to bile salt caused dissolution and enzymatic degradation in the gastro-intestinal tract, however, remains as the main barrier for their beneficial utilization in oral delivery (Childers and Michalek, 1994). Therefore, to exploit the full potential of liposomes as immunological carriers for oral delivery we have developed and evaluated stable liposomal adjuvant utilizing a novel technique reported by Sunamoto et al. (1984).

The aim of the work was to synthesize and characterize a modified polysaccharide, *O*-palmitoylpullulan (OPP) and to use it for coating of the unilamellar vesicles containing the model antigen, bovine serum albumin (BSA). The liposomes so prepared were characterized for their size, shape, stability and their antibody stimulating efficiency following oral administration.

2. Materials and methods

2.1. Materials

Egg phosphatidylcholine (PC), cholesterol,

Table 2
Formulation code with varying polysaccharide to lipid ratio and their ζ potential values

Formulation code	OPP:lipid (w/w)	Size (μm)	ζ Potential (mV)
PC1	0:10	2.5 ± 0.63	88.5 ± 6.0
PC2	1:9	2.80 ± 0.59	65.5 ± 4.0
PC3	2:8	3.25 ± 0.74	40.2 ± 5.0
PC4	3:7	3.8 ± 0.68	18.0 ± 3.0
PC5	4:6	4.1 ± 0.54	4.5 ± 1.0

Table 3
Mean vesicle size and number of vesicles before and after incubation in SGF for 3 h

Formulation code	Mean vesicle size (μm)		No. of vesicles per cubic millimetre (No. $\times 10^3$)	
	Before incubation	After incubation	Before incubation	After incubation
PC5	4.1 ± 0.54	4.8 ± 0.38	47	46
P2	2.5 ± 0.63	5.3 ± 0.47	50	42

Table 4
IR spectral data of OPP

Observed values (cm^{-1})	Expected values (cm^{-1})	Functional group	Interpretation
1685	1750–1735	C$O	Carbonyl band stretching
3460	3600–3200	Free–OH	Stretching vibration of O–H bond
2940	2960–2850	CH_2CH_3	C–H stretching
1100	1150–1040	C–O	C–O stretching

phosphatidylethanolamine (PE), BSA and pullulan were obtained from Sigma. Palmitoyl chloride from Fluka Chemika. All other chemicals and reagents were obtained from Merck, India, and used as supplied.

2.2. Synthesis and characterization of O-palmitoylpullulan

O-palmitoylpullulan (OPP) was prepared by a method described earlier by Sunamoto et al. (1992). Briefly, 1 g pullulan was dissolved in 11 ml dry dimethylformamide at 60°C. To the resulting solution, 1 ml dry pyridine and 0.1 g palmitoyl chloride, dissolved in 0.24 ml dry dimethylformamide, were added. The mixture was stirred at 60°C for 2 h followed by 1 h at room temperature. This mixture was then slowly poured into 70 ml absolute ethanol under stirring. The precipitate so formed was collected and washed with 80 ml absolute ethanol and 60 ml dry diethyl ether. The white solid material obtained was dried in vacuum at 50°C for 2 h.

The synthesized polymer was characterized by IR and ^{1}H NMR. The IR spectrum of OPP (1%) was taken by incorporating it into a compressed KBr pellet. Similarly, a ^{1}H NMR spectrum was obtained in deuterated dimethylsulfoxide solution (DMSO-d_6) using tetramethylsilane (TMS) as internal standard. Additionally, a ^{1}H NMR spectrum of pullulan was also obtained.

Table 5
Proton NMR resonance spectral data of OPP

Observed values (ppm)	Expected values (ppm)	Functional group	Interpretation
0.858	0.9	$-CH_3$	Terminal methyl group
1.20	1.17	$-(CH_2)_{12}$	Methylene group
1.234	1.4	$-CH_2\beta$	β–Methylene group
2.38	2.3	$-CH_2\alpha$	α-Methylene group
4.647	4.54	–H	At C_1 position of α1,6 glycosidic bond
4.996	5.04	–H	At C_1 position of β1,6 glycosidic bond
2.546–3.568	2.60–4.20	–H	At C_2–C_6 positions glucose residues

2.3. Preparation of liposomes

Liposomes were prepared by the reverse phase evaporation technique as reported by Sazoka and Papahadjopoulos (1978) with slight modifications. Briefly, PC, cholesterol and PE were taken in different mole fractions (Table 1) and dissolved in 5 ml diethylether to which 2 ml of aqueous phase, i.e. phosphate buffer saline (PBS) (pH 7.4) containing 2 mg BSA was added. The mixture was sonicated (Soniweld) for 10 min (with an interval of 2 min after 5 min). A thick emulsion was formed which was then kept over a vortex mixer in order to remove any residual ether. To this emulsion 3 ml warm PBS (pH 7.4) was added in order to hydrate the vesicles.

2.4. Encapsulation efficiency

The prepared liposomes were taken and the free (unentrapped) antigen was separated by a Sephadex G-25 minicolumn using centrifugation technique (Fry et al., 1978). The method was repeated thrice with a fresh syringe packed with gel each time. The fraction was finally collected which was free from unentrapped antigen.

The isolated liposomes were centrifuged at 60 000 rpm for 4 h. The pellets were challenged with Triton X-100 (0.2%) to disrupt the vesicles and the liberated antigen was estimated by the Bradford method (Bradford, 1976). The percent fraction of antigen entrapped was calculated and recorded (Table 1).

2.5. Coating of liposomes

OPP was dissolved in 2 ml PBS (pH 7.4). The liposomes were coated with OPP by adding 2 ml of solution to 4 ml of liposome dispersion under constant stirring. Stirring was continued for 1 h in order to ensure complete coating of the liposome (Sunamoto et al., 1987) (photomicrograph 1).

2.6. Measurement of ζ potential

The movement of a charged surface with respect to an adjacent liquid phase is the basic principle underlying this technique. Malvern zeta-

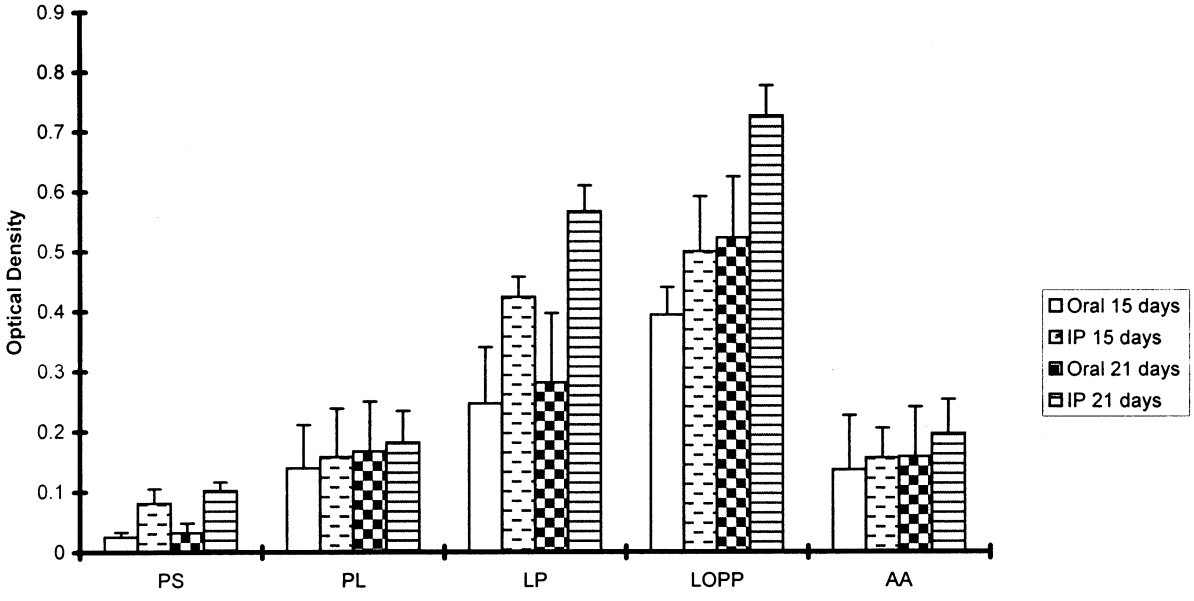

Fig. 1. Serum IgG levels in rats following oral and IP administration of various formulations.

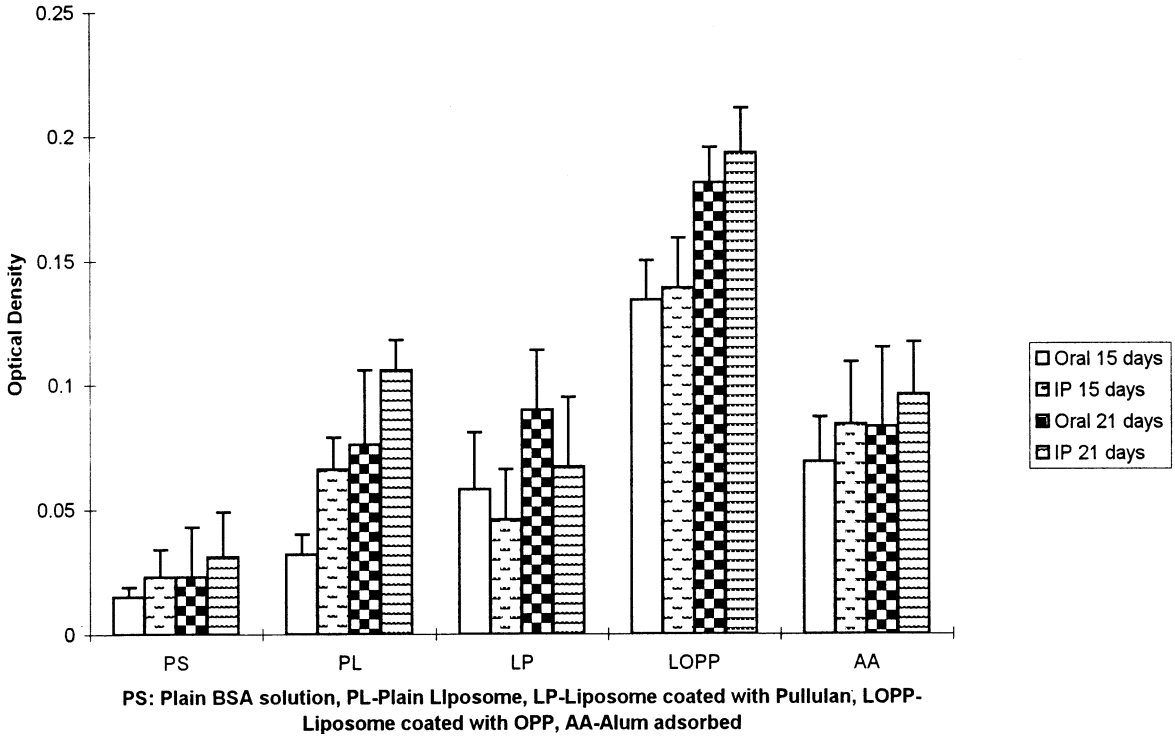

Fig. 2. Serum IgA levels in rats following oral and IP administration of various formulations.

sizer was used in the present study to calculate the ζ potential. The results are as recorded in Table 2.

2.7. Measurement of size

Mean particle size of liposomes was measured by photoncorrelation spectroscopy with an Autosizer II C apparatus (Malvern Instruments, UK).

2.8. Stability in simulated gastric fluid (pH 1.5)

The present study involves the delivery of OPP-coated liposomes via the oral route. Hence, it was necessary to study stability of the vesicle in simulated gastric fluid (SGF). Coated and uncoated liposomal suspension (1 ml) was taken in two separate dialysis tubes (Sigma, USA) and was placed in a beaker containing 250 ml SGF. The beaker was placed over a magnetic stirrer and stirred continuously for 3 h. The mean vesicle size and number of vesicles per cubic millimetre before placing in the SGF and 3 h after placing in SGF was determined. The results are as recorded in Table 3.

2.9. Immunization protocols

Ten groups of albino rats (Wistar strain) weighing 120–150 g were used with three rats in each group. On day 1 groups of rats were administered orally with preparations containing BSA equivalent to 100 μg. A similar group of rats was injected intraperitoneally. Secondary immunization was done on day 15 with formulations containing BSA equivalent to 100 μg. Blood was collected from the orbital plexus on day 15 and 21 and the production of anti-BSA IgG and IgA antibody in the serum was quantified.

2.10. Measurement of IgG and IgA by ELISA

Specific anti-IgG and IgA antibody levels in the serum were determined by ELISA using a slightly modified method of Michalek et al. (1989). Each

well of the microplate (Dynatech) was coated with 50 μl of a BSA (100 μg ml^{-1}) solution in 50 mM carbonate buffer (pH 9.6) at 4°C overnight. After being washed three times with 10 mM PBS-buffered saline containing 0.05% Tween 20 (PBS–Tween), the wells were coated with 100 μl 3% skim milk in PBS–Tween for 1 h at room temperature to block non-specific adsorption of the antibodies. After washing with PBS–Tween diluted serum (50 μl per well) was added. Following incubation at 25°C for 2 h and washing, horseradish peroxidase-conjugate antibodies having specificity for rat IgG or IgA (Sigma) diluted with PBS–Tween (500 ng ml^{-1}) were added (50 μl per well). The plates were incubated at 25°C for 2 h then washed and 50 μl 0.015% H_2O_2 containing 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (15 μg ml^{-1}) dissolved in 0.1 M citrate buffer (pH 4.0) were added to each well. The wells were incubated at 37°C for 20 min, absorbance at 405 nm was measured with a microplate reader (Molecular device).

3. Results and discussion

3.1. Characterization of OPP

Pullulan, a naturally occurring polysaccharide produced by the yeast like fungus *Pullalaria pullulans*, is known to protect plasma membranes against physicochemical stimuli such as osmotic pressure and ionic strength. However, when adsorbed on to the liposomal surface it is easily removed on dilution. Therefore, it was necessary to chemically modify pullulan by conjugating it to a hydrophobic group which allows the polysaccharide to intereligitate with the liposomal membrane (Sato and Sunamoto, 1992). The, pullulan used in the present study was chemically modified by esterification with palmitoylchloride. The resultant product was characterized by IR and ^1H NMR spectrum. The results are presented in Table 4.

Characterization of OPP by IR spectroscopy helped in the identification of carbonyl groups. Pavia et al. (1979) reported that a characteristic stretching vibration is observed at about 1735 cm^{-1} due to the carbonyl (C$O) bond. However, here in the synthesized OPP the stretching vibration was observed at 1685 cm^{-1}. This shift in frequency may be attributed to the intermolecular hydrogen bonding between the carbonyl and hydroxyl group. The presence of hydrogen bonds was confirmed from the OH stretching vibration at 3460 cm^{-1}. This represents a polymeric band. However, there is a shift in band from 3600–3200 cm^{-1} to 3460 cm^{-1} which may be attributed to the intramolecular single bridge hydrogen bonds and the intermolecular bridge between hydrogen bonds. A characteristic C–H stretching vibration was observed at 2940 $cm^{-1}$and a C–O stretching at 1100 cm^{-1} From these observations it can be concluded that there exists a ester bond between pullulan and palmitoyl residues suggesting palmitoylation of pullulan.

The OPP formation was further confirmed by ^1H NMR resonance spectroscopy (Table 5). The protons corresponding to the terminal methyl group of the palmitoyl chain were observed at 0.850 ppm, that of the 12-methylene groups were observed at 1.20 ppm while those at 1.234 and 2.38 ppm were indicative of the presence of β and α methylene groups, respectively. These observations were in accordance with those found by Moreira et al. (1997). Two peaks at the down field at 4.996 and 4.647 ppm were observed which indicated the C_1 position of α1,4 and α1,6 glycosidic bonds, respectively. It was possible to identify a range of protons from 2.546–3.568 ppm corresponding to the glucose residue units at positions C_2–C_6. This observation was in accordance with Akiyoshi et al. (1990) where they reported a range of 2.60–4.20 ppm. From these observed data the formation of OPP was further confirmed.

3.2. Characterization of liposomes

It is apparent from Table 1 that the variation in phospholipid ratio used in the preparation of liposomes demonstrated a significant difference in their size and encapsulation efficiency. Encapsulation efficiency in the case of P1 was found to be $37.5 \pm 0.9\%$, while for P2 and P3 it was 40.3 ± 1.8 and $35.6 \pm 1.2\%$, respectively. The low percent encapsulation in the case of formulation P1 may

be due to the formation of a leaky vesicle, as the cholesterol content in this formulation was relatively less. Similarly, a lower encapsulation value was obtained with formulation P3. Here the cholesterol content was high enough, probably beyond its optimum concentration ratio, to result in packing with decreased fluidity. Similarly, the vesicle size in the cases of P1 and P3 showed a great deal of variation with a high dispersibility index indicative of heterogeneity in the liposome population. The wide range of vesicle size in the case of P3 (5.4–8.5 μm) may be due to the reduction in area which might have arisen due to condensation of the membrane. In the case of P1 the vesicle size was found to range between 4.3 and 5.4 μm. This may be due to the formation of leaky, less rigid vesicles leading to fusion of the vesicles contributing larger vesicles. From the three formulations prepared as mentioned in Table 1, formulation P2 was chosen for further coating of the liposomes with OPP, where the cholesterol ratio was considered to be at the optimum level (PC:CH:PE, 7:2:1).

3.3. Coating of liposomes

Based on encapsulation efficiency (40.3%) and size (2.5–3.35 μm), formulation P2 was selected for further studies. The liposomes were coated using OPP. The process of coating liposomes with OPP was optimized by varying the polysaccharide:lipid ratio (Table 2). The coating of liposomes was indirectly measured using a Malvern zetasizer. The observations as noted from Table 2 indicate that on increasing the polysaccharide ratio (w/w) from 1 to 4 there was a decrease in ζ potential by 88.5 ± 2.5, 65.5 ± 1.5, 40.0 ± 2.5, 18.0 ± 3.0 and 4.5 ± 1.5 mV for formulations PC1 to PC5, respectively. From these results it can be concluded that the initial, high ζ potential values are ascribed to the charge on the surface of the liposomes imparted by phosphatidylethanolamine. On addition of the negatively charged OPP, the charge on the liposomes is neutralized due to the charge induced coating of the negatively charged OPP on the positively charged liposomes. The resultant fall in ζ potential was found to be very significant in the case of formulation PC4. On further increasing the OPP concentration the ζ potential approached a near zero value. This is an indication of the almost complete intrinsic charge of quenching on coating of the liposomes with OPP. Hence, the formulation PC5 with polysaccharide:lipid ratio (w/w) of 4:6 was selected as an ideal one and used in further studies. The coated vesicles did not show any significant increase in vesicle size (Table 2). The presence of coating was seen as shown in Fig. 3.

3.4. Stability in SGF

The mean vesicle size of uncoated liposomes (P2) was 2.5 ± 0.63 μm and that of the coated liposomes (PC5) was 4.1 ± 0.54 μm before incubation in SGF. The number of vesicles per cubic millimetre were 50×10^3 in the case of P2 and 47×10^3 in the case of PC5. The mean vesicle size increased dramatically from 2.5 to 5.3 μm in the case of formulation P2 which was further confirmed by the fall in the number of vesicles per cubic millimetre. However, the mean vesicle size as well as the number of vesicles per cubic millimetre did not show any significant change in case of formulation PC5 following their incubation in SGF for a period of 3 h. This clearly indicated that the polymer coated vesicles are stable in SGF.

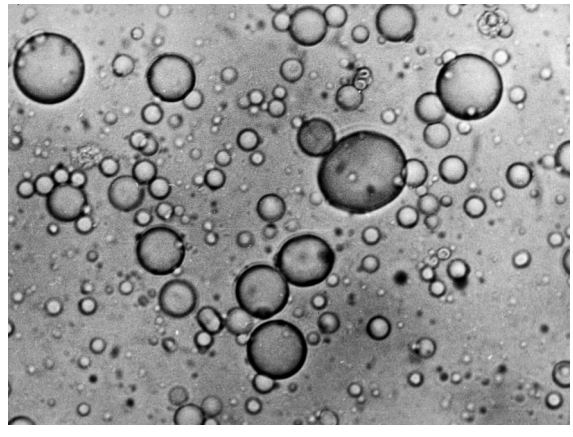

Fig. 3. OPP coated liposomes (× 125).

3.5. Systemic IgG and IgA responses

Rats which were inoculated with BSA in liposomes coated with OPP by oral administration exhibited an increased serum IgG and IgA titres against BSA in other forms (Figs. 1 and 2). The antibody titre values recorded as optical density (O.D.), following oral administration of liposomes coated with OPP, was found to be 0.134 and that after intraperitoneal administration, it was 0.139. This shows that there was no significant effect dictated by the route of administration on secretion of IgA. However, a significant change in IgG levels due to the route of administration was observed. Serum IgG level on day 15, in the case of orally administered OPP coated liposomes, were found to have an O.D. of 0.394 while that administered via the intraperitoneal route showed an O.D of 0.500. Antibody levels on administration of liposomes coated with pullulan alone did elicit an immune response better than plain liposomes. This may be attributed to the pullulan, that it possesses some antigenicity (Moreira et al., 1997). The pullulan borne antigenicity may lead to an increase in serum IgG levels. However, the serum IgA level recorded was lower compared to its palmitoyl derivative coated liposomes.

The increased IgA level obtained following oral administration of OPP coated liposomes compared to plain liposomes or pullulan coated liposomes signifies that the OPP coated liposomes are stable even after passing through the stomach and might be taken up by the Peyer's patches.

In this study we observed that there was a significant level of IgA secretion following primary immunization, whilst earlier reports using biodegradable microspheres for oral immunization showed a significant IgA secretion only after secondary immunization (Challacombe et al., 1992). Even though biodegradable microspheres have been studied exhaustively for oral immunization the method involved in the preparation of these microspheres may lead to a loss of the antigenicity. To overcome this alginate microspheres are being studied (Bowersock et al., 1996; Cho et al., 1998). However, the control of microsphere size below 10 μm which renders them capable of passing through Peyer's patches is of importance. Liposomes used in the present study were in the size range 2.5–3.35 μm and as a result might have been responsible for their uptake by the Peyer's patches. In addition, liposomes are protected from acids, proteolytic enzymes, lipases and bile salts by the OPP coating (net). Liposomes as an immunoadjuvant for mucosal immunization have been studied by several groups (Childers and Michalek, 1994; Kersten and Crommelin, 1995; Michalek et al., 1995). However, their stability was a cause of concern which we have tried to alleviate by coating them with OPP. Moreover, further studies are being carried out to elucidate whether the coated liposomal systems are capable of stimulating antibody clones over a prolonged period of time.

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