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Lectin anchored stabilized biodegradable nanoparticles for oral immunization 1. Development and in vitro evaluation

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Abstract

The investigation comprises development of a stable and targeted formulation of HBsAg for the oral immunization against Hepatitis B. PLGA nanoparticles bearing HBsAg was prepared by double emulsion method. The antigen was protected from organic/aqueous interface by using protein stabilizer, trehalose. The acidic environment generated within PLGA nanoparticles was neutralized by co-encapsulation of a basic additive, $Mg(OH)_2$ which provides an additional stabilization to the antigen especially against acid induced antigen inactivation. Furthermore, lectin from *Arachis hypogaea* (PNA) was anchored on to the surface of the HBsAg loaded nanoparticles in order to enhance their affinity towards the antigen presenting cells of the Peyer's patches. The developed system was characterized for shape, size and loading efficiency. The antigen integrity was assessed by using SDS-PAGE followed by isoelectric focusing analysis. Bovine submaxillary mucin (BSM) was used as a biological model for in vitro ligand affinity determination and activity studies. The lectin anchored nanoparticles exhibited $52.18 \pm 4.73\%$ loading while ligand density was estimated to be of 17.90 ± 1.14 μg/mg. The results suggest that HBsAg can be successfully stabilized by co-encapsulation of an appropriate protein stabilizer, i.e. trehalose and a basic additive, $Mg(OH)_2$. The ligand-coupled nanoparticles demonstrated approximately four folds increase in degree interaction with the BSM as compared to plain nanoparticles. Additionally, the nanoparticles maintained their intrinsic sugar specificity as associated due to lectin (PNA).

Keywords: Oral immunization; HBsAg; Protein stability; Vaccine targeting; Nanoparticles; *Arachis hypogaea*

1. Introduction

Mucosal membranes are exposed to antigenic substances that can induce specific humoral and cell mediated immune responses. Albeit immunization via mucosal surfaces can induce both mucosal as well as systemic immunization, mucosal responses are generally not observed following systemic immunization. Oral vaccine delivery is associated with numerous potential advantages viz. improved safety, patient compliances and acceptance and reduced cost, however peroral antigen delivery is confronted with several challenges. The protection of structural integrity of antigen until it reaches the predetermined target site is the major challenge, which is created by the hostile gastrointestinal environment (Gabor et al., 2002; Venkateshan and Vyas, 2000; Singh et al., 2004). Harsh gastrointestinal conditions and poor immunogenicity of many purified antigen generally render oral vaccine delivery ineffective (Lavelle et al., 2004). Various strategies and delivery systems have been devised for effective oral vaccine delivery. Microparticles/nanoparticles delivery systems are particularly useful to protect the antigen in the gastrointestinal tract and hold potential to enhance the efficacy of oral vaccine (O'Hagan, 1998). Polymerized liposomes, which are having improved stability in gastrointestinal tract, have also been exploited for oral immunization (Chen et al., 1996). Enhancing the specific binding of particulates to intestinal mucosal using some cell selective ligands and subsequent translocation/uptake by cells of gastrointestinal lining is another approach for the effective oral vaccine delivery (Lavelle et al., 2001). Recently mannosylated niosomes are documented in our laboratory as an adjuvant carrier system for the oral genetic immunization against Hepatitis B (Jain et al., 2005).

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The majority of gut-associated lymphoid tissue (GALT) is organized into aggregates of lymphoid follicles called as Peyer's patches. Antigens, particulate carriers, bacteria, viruses and protozoa are transported into Peyer's patches by specialized epithelial cells called M cells (Owen and Ermark, 1990). The uptake and transport of antigens by M cells is related to two aspects; first, antigen will probably escape degradation and secondly the antigen will be released into an environment rich in immunocompetent cells. Therefore, the uptake by M cells allows the delivery of intact antigen into the immune-inductive environment of the Peyer's patches and restricts the access of antigens to alternative areas of the intestine, where suppressor T cells predominate. Hence, antigen after being taken up by M cells induces potent immune responses whereas the antigens that are taken up by ordinary enterocytes are more likely to induce systemic tolerance. Thus M cells targeted oral vaccine delivery systems hold promise to improve the efficacy of the oral vaccines.

The incorporation of proteins in the PLGA microparticles/nanoparticles suffers significant protein degradation during preparation, storage and following in vivo administration. Among the various strategies to prevent interface-induced protein denaturation and aggregation, the addition of polyol or sugar excipients in the aqueous phase is well documented (Cleland and Jones, 1996; Perez and Griebenow, 2001). Further, acidity commonly develops in PLGA formulations because of accumulation of acidic degradation products upon polyester hydrolysis (Shenderova et al., 1999; Brunner et al., 1999) and the acidic microenvironment of PLGA delivery systems is a potential cause of instability of encapsulated proteins.

Oral vaccination is largely ineffective due to substantial degradation of antigens by gastric acid and proteolytic enzymes. Furthermore, poor absorption of the antigens by the gut-associated lymphoid tissue requires larger doses of antigen to be administered to achieve desirable level of immunity comparable with systemic administration. Non-living carriers (micro/nanoparticles, liposomes, etc.) have been extensively used for vaccine delivery. Particulate oral vaccines that depend on non-specific interactions for transport into mucosal inductive sites can be effective in evoking mucosal immune responses but they require very large oral doses. Thus it seems likely that vaccine efficacy could be enhanced by anchoring M cells specific ligands (e.g. lectins) on to the bioactive carriers (Chen et al., 1996; Gupta et al., 2005). Among the various approaches used to enhance oral delivery of bioactive we have exploited lectins for the targeted oral immunization. Lectins are proteins or glycoproteins capable of specific recognition of and reversible binding to carbohydrate determinants of complex glycoconjugates, without altering the covalent structure of any of the recognized glycosyl ligands. Lectin receptors are expressed on various cells such as endothelial cells, hepatocytes, macrophages, monocytes and lymphocytes. They are efficient in recognizing the complex oligosaccharide epitopes, which are also present on the cell surface or could be exogenous glycoconjugate ligands mimics of endogenous carbohydrate epitopes (Vyas et al., 2001a).

In the present investigation, PLGA nanoparticles encapsulating HBsAg were developed. The antigen in the nanoparticles was stabilized by using protein stabilizer, trehalose. Further, an attempt has been made to co-encapsulate a basic additive, $Mg(OH)_2$ which stabilizes the antigen through neutralizing the acidic microclimate pH in the polymer (generated within the nanoparticles owing to the degradation of PLGA). Additionally, to improve oral vaccine efficacy, the nanoparticles were coupled with M-cell targeting ligand *Arachis hypogaea* (Clark et al., 1993) and targeting potential was assessed by bovine submaxillary mucin assay.

2. Materials and methods

2.1. Materials

PLGA with a lactide to glycolide ratio of 50:50 (MW 40,000–75,000 Da), polyvinyl alcohol (MW 30,000–70,000 Da), *A. hypogaea* (PNA), FITC-conjugated lectin (f-PNA), pepsin (2500–3500 U/mg), trypsin (10,000 BAEE U/mg) and bovine submaxillary mucin were procured from Sigma Chemical Co. (St. Louis, MO, USA). Glutaraldehyde (25% in water) was purchased from Fluka Chemica Co. (AG CH-9470 Buchs, Switzerland). HBsAg (MW 24 kDa) was obtained from Panacea biotech Ltd. (Lalru, Punjab, India). BCA protein estimation kit and protein molecular weight markers were purchased from Genei, Bangalore, India. All other chemicals and reagents were of analytical grade.

2.2. Preparation of PLGA nanoparticles

PLGA nanoparticles were prepared by double emulsion method as reported by Davada and Labhasetwar (2002) with modifications. Briefly, to the 1 ml aqueous phase 150 μl of recombinant HBsAg, 1.5% (w/v) trehalose and 2% (w/v) $Mg(OH)_2$ was added which was further suspended in 10 ml of 4% (w/v) PLGA in dichloromethane. The mixture was probe sonicated (Soniweld, India) for 60 s at 40 W in an ice bath. To this water-in-oil emulsion, 40 ml of 5% (w/v) aqueous polyvinyl alcohol was added and probe sonicated for 3 min to obtain a w/o/w emulsion. The emulsion was stirred vigorously for 3 h. The nanoparticles were collected by centrifugation, washed twice with distilled water to remove PVA and then lyophilized.

2.3. PVA determination

The colorimetric method described by Joshi et al. (1979) was used to quantitate the amount of PVA associated with the nanoparticles. In brief, 5 mg of washed and lyophilized nanoparticles were hydrolysed in 2 ml of 0.5N sodium hydroxide solution for 15 min at 60 °C. The solution was neutralized with 900 μl of 1N HCl and volume was made up to 5 ml with milli-Q water. Three milliliters of a 0.65 M solution of boric acid, 0.5 ml of a solution of I_2/KI (0.05 M/0.15 M) and 1.5 ml of milli-Q water were added to the neutralized solution and absorbance of the solution was determined at 690 nm after a

15 min incubation (Shimadzu-1604, UV–vis spectrophotometer, Japan).

2.4. Assessment of lectin integrity in GIT

To assess the gastrointestinal stability equal volume of the lectin (0.3% (w/v) in saline) and 0.25% (w/v) pepsin in 0.1 M glycine–HCl (pH 2.5) and 0.25% trypsin in 0.1 M Tris–HCl (pH 7.9) were incubated at 37 °C. Aliquots were drawn in regular intervals up to 24 h and analysed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE, Bio-Rad, USA). The gels were stained with silver staining solution. Bovine serum albumin (0.15% (w/v) in phosphate buffer saline) was used as a positive control for proteolytic activity of the enzyme used.

2.5. Preparation of lectin coupled PLGA nanoparticles

Lectin (PNA) was covalently coupled to PVA associated to the surface of nanoparticles by method as reported by Montisci et al. (2001) with slight modification. Method involves two steps: activation of hydroxyl group of surface associated PVA followed by its coupling with lectin.

2.5.1. Activation of hydroxyl group of nanoparticle by using glutaraldehyde

Fifty milligrams of the nanoparticles were washed in milli-Q water by centrifugation (20,000 × *g*, 15 min). The pellet was resuspended by vortexing in 2 ml of milli-Q water and 1 ml of glutaraldehyde (25% aqueous solution) and 250 μl of 0.3 M H_2SO_4 were added. The mixture was then shaked gently for 1 h at 30 °C to activate hydroxyl group of surface anchored PVA, a longer time lead to aggregation.

2.5.2. Conjugation of nanoparticles with lectins

The unreacted glutaraldehyde was removed by centrifugation. Any remaining traces of glutaraldehyde were removed by three washing in phosphate buffer saline (PBS 10 mM, pH 7.4) to remove any traces of glutaraldehyde, which might otherwise cross-link the lectin molecule. Then 900 μl of PBS containing 250 μg of lectin was added for surfacial conjugation by incubation overnight at room temperature. The conjugates were centrifuged to remove free lectins and incubated 1 h with ethanolamine (0.1 M) to mask unreacted groups on the particles. The ethanolamine was removed and nanoparticles were washed three times by centrifugation. The lectin-coupled nanoparticles were finally resuspended in 1 ml PBS and stored at 4 °C.

2.6. Determination of the amount of bound lectin

The amount of lectin coupled to nanoparticles was estimated and determined as the difference between the lectin added initially and the lectin recovered in the solution after incubation with the particles. The amount of lectin was quantified by the colorimetric determination of protein in the supernatant by bicinchoninic protein assay (BCA protein kit, Genie, Bangalore).

2.7. Stability of surface modified nanoparticles

The method reported by Ertl et al. (2000) was used to investigate the stability of conjugation between lectin and PLGA nanoparticles. FITC labeled PNA (f-PNA) was used for the surface anchoring with PLGA nanoparticles. Ten milligrams of f-PNA coupled nanoparticles were mixed with 1 ml of HEPES buffer at pH 7.4 at 4 °C. The supernatant was analysed at regular intervals by using spectrofluorimeter (SPECTRA max GEMINI XPS, Molecular Device) after centrifugation at 22,000 × *g* for 20 min at 4 °C and filtration through 0.45 μm filters. The aliquot removed was replaced by fresh HEPES buffer.

2.8. Morphology and particle size analysis

The nanoparticles were observed for their surface morphology by scanning electron microscopy (SEM, JEOL 6100, Japan). The nanoparticles were placed on the sample holders, sputter coated with gold and then placed in scanning electron microscope. The mean diameter of the nanoparticles was determined by Autosizer II C apparatus (Malvern Instruments, UK).

2.9. Protein loading efficiency

The loading efficiency of the HBsAg in the PLGA nanoparticles was determined by dissolving 20 mg the nanoparticles in the 2 ml of 5% (w/v) sodium dodecyl sulfate in 0.1 M sodium hydroxide solution (Singh et al., 1997). The amount of the antigen was determined by micro-bicinchoninic acid assay. Similarly, protein content of lectin-coupled HBsAg loaded nanoparticles (*a*) and lectin-coupled placebo nanoparticles (without antigen) (*b*) was determined. The difference of two values i.e. $(a - b)$ represent the amount of HBsAg loaded with the lectin anchored nanoparticles.

2.10. In vitro release of HBsAg

The in vitro release of HBsAg from PLGA nanoparticles was carried out in PBS (pH 7.4). Vials containing 40 mg of nanoparticles and 5 ml of PBS (pH 7.4) were incubated at 37 °C on a constant shaking mixer. Tween-80 (0.02%, w/v) was added to the release media to reduce the absorption of the released protein as well as to prevent the particles from clumping and to improve their wettability. At appropriate intervals 1.0 ml of release medium was collected following centrifugation at 22,000 × *g* for 20 min and 1.0 ml of fresh PBS (pH 7.4) was again added to the vial. The amount of HBsAg released was estimated by AUZYME monoclonal kit (Abbott Laboratories, Abbott Park, IL, USA). The same sample was used to measure in vitro antigenicity using an enzyme immunoassay (EIA) kit (AUSZYME; Abbott Laboratories, Abbott Park, IL, USA) as described by Shi et al. (2002). The in vitro antigenicity of HBsAg was evaluated by using the ratio of the EIA response to protein concentration (EIA/protein).

The neutralization effect of $Mg(OH)_2$ on the acidic microenvironment was investigated by incubating the nanoparticles (5 mg) at 37 °C for 4 weeks and the pH was measured. The degradation half-life of PLGA nanoparticles was also determined by gel permeation chromatography. Average molecular weights were calculated using a series of polystyrene standards as described by Barrera et al. (1995).

2.11. Evaluation of protein (HBsAg) aggregation

Protein may aggregate on exposure to adverse encapsulation conditions; therefore percent aggregation was determined by method described by Zhu et al. (2000). In brief, incubated polymers were removed from release medium, dried and dissolved in acetone. After centrifugation and removal of the polymer solution, the remaining HBsAg pellet was reconstituted in PBS (pH 7.4) containing 0.2% Tween-20 (PBST) and incubated (37 °C) overnight before determining the protein content. This gave a measure of the water-soluble protein encapsulated. Any aggregates were collected by centrifugation and incubated (37 °C for 30 min) in denaturing solvent (PBST, 6 M urea, 1 mM EDTA). The non-covalently bonded HBsAg aggregates were analysed and determined for protein content.

2.12. Assessment of structural integrity of HBsAg

The structural integrity of HBsAg extracted from the nanoparticles was detected by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and compared with the native HBsAg and reference markers (Laemmli, 1970; Singh et al., 1997). HBsAg was extracted by dissolving the nanoparticles in 2 ml of 5% (w/v) SDS in 0.1 M sodium hydroxide solution (Singh et al., 1997). The extracted antigen was concentrated and loaded onto a 3.5% stacking gel and subjected to electrophoresis on a 12% separation gel at 200 V (Bio-Rad, USA) until the dye band reached the gel bottom. After migration the gel was stained with coomassie blue to reveal the antigen, which was then destained and dried. To examine potential structural alterations of encapsulated HBsAg, isoelectric focusing (IEF) was performed to characterize the loaded antigen (Zhu et al., 2000).

2.13. In vitro ligand affinity and activity studies

The activity of the nanoparticles coupled with PNA towards exogenously provided bovine submaxillary gland mucin (BSM) and affinity toward competing sugar were studied to assess the targeting efficacy of ligand-anchored nanoparticles. The in vitro targeting potential was determined by mixing 1 ml of BSM in PBS (0.5 mg/ml) and same volume of suspension of PNA coupled nanoparticles in PBS. After optimum incubation (60 min) samples were centrifuged at 15,000 rpm for 10 min, the aliquots of the supernatant were taken and 20 μl was injected into the HPLC system (Ezpeleta et al., 1996). The amount of interacted BSM was calculated as difference between the total and the remaining BSM in the clear supernatant. To study specificity, D-galactose was added to the BSM bulk solution in PBS and interaction of PNA coupled nanoparticles and BSM was determined.

2.14. Statistical analysis

The results were presented as mean ± standard deviation. Statistical analysis was carried out using Student's *t*-test and statistical significance was designated as $P < 0.05$.

3. Results and discussion

3.1. Assessment of lectin integrity in gastro-intestinal tract

For a lectin to be useful as a targeting ligand it must have stability against enzymatic attack in the gastrointestinal tract. Enzyme has a characteristics pH at which their activity is maximal. Pepsin, which hydrolyses certain peptide bonds of protein during digestion in the stomach, has optimum pH in the acidic range and the activity optima of the pancreatic enzyme e.g. trypsin occur at pH 7–8 (Lehninger et al., 1993). Thus buffer of pH 2.5 and 7.9 were chosen for the optimum activity of the enzyme pepsin and trypsin, respectively. This is also in accordance of the report of Gabor et al. (1997) dealing with proteolytic degradation of lectins. The enzymes selected for the assessment of proteolytic degradation in this study mimic the gastro-intestinal environment as they cover the spectrum of proteolytic activity present in the gastro-intestinal tract. Further, the amount of enzymes used and the incubation time was relatively higher than those found under physiological conditions thus lectin was expected to degrade than would occur otherwise in vivo. The SDS-PAGE analysis of the lectin *A. hypogaea* revealed negligible to no degradation of product (Fig. 1) thus the lectin could resist the proteolytic attack specifically in for-

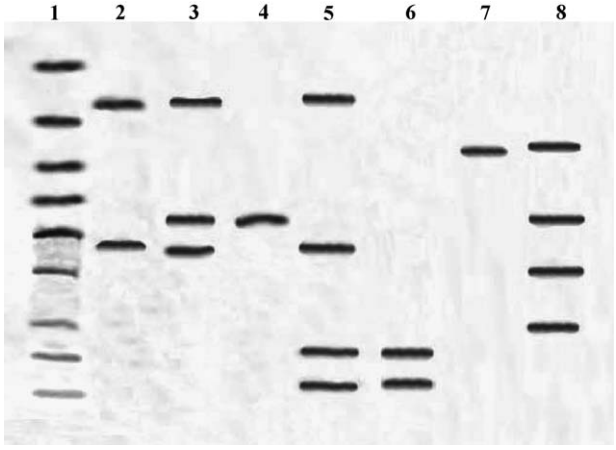
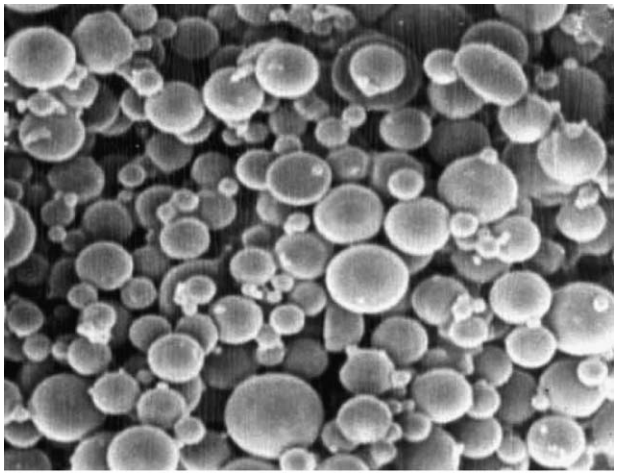
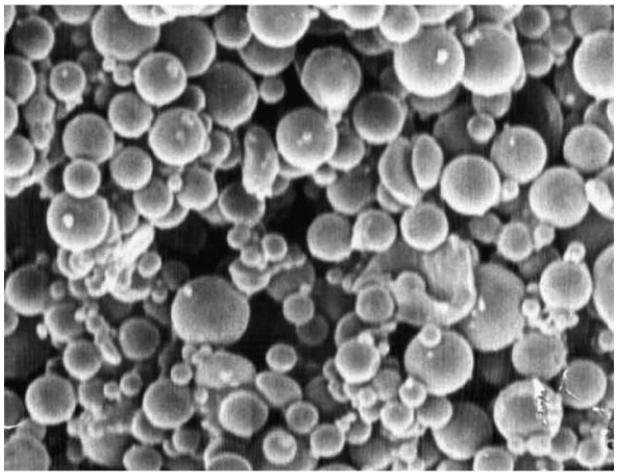


Fig. 1. SDS-PAGE analysis of *Arachis hypogaea* (PNA) after incubation with pepsin or trypsin for 3 h at 37 °C. Lane 1: marker proteins; 205 kDa myosin, rabbit muscle; 97.4 kDa phosphorylase b; 66 kDa bovine serum albumin; 43 kDa ovalbumin; 29 kDa carbonic anhydrase; 20.1 kDa soyabean trypsin inhibitor; 14.3 kDa lysozyme; 6.5 kDa aprotinin; 3 kDa insulin (α and β chains). Lane 2: PNA (0.15%). Lane 3: PNA (0.15%) with pepsin (0.25%). Lane 4: pepsin (0.25%). Lane 5: PNA (0.15%) and trypsin (0.25%). Lane 6: trypsin (0.25%). Lane 7: BSA (0.15%). Lane 8: BSA (0.15%) and pepsin (0.25%).



(A)



(B)

Fig. 2. Scanning electron micrograph of plain nanoparticles (A) and lectin anchored nanoparticles (B) loaded with HBsAg.

mulations and considered to be a useful ligand for the targeting of nanoparticulate systems to the antigen presenting cells of the Peyer's patches.

3.2. Characteristics of PLGA nanoparticles

The PLGA nanoparticles were prepared by double emulsion method. The plain PLGA nanoparticles were spherical in shape with smooth surfaces as visualized by SEM (Fig. 2). The loading efficiency of HBsAg-PLGA nanoparticles was 54.51 ± 4.80% and their average particle size was measured to be 390.72 ± 20.38 nm. Characteristics of plain and lectinized nanoparticles were compared in Table 1.

Table 2
Effect of washing by ultrafiltration on mean diameter and residual PVA concentration of PLGA nanoparticles

Number of ultrafiltration	Mean diameter (nm)	PVA concentration (%)
2	399.54 ± 15.27	4.14 ± 0.31
4	392.71 ± 11.63	3.98 ± 0.35
6	389.42 ± 24.36	3.94 ± 0.46
8	389.58 ± 19.58	3.93 ± 0.27

Data represent mean ± S.D. ($n = 4$).

3.3. Characteristics of lectin-coupled PLGA nanoparticles

PLGA nanoparticles were coupled with lectin PNA to confer on them M-cell targeting potential and to make them antigen carrier for oral immunization. Different strategies can be used to couple ligands to the surface of the nanoparticles. Irache et al. (1994) reported covalent coupling of lectin to amino- or carboxylic groups present on the surface of polystyrene nanoparticles. In the present investigation hydroxyl group of the PVA at the surface of the nanoparticles were used for the conjugation of lectins. A two-step procedure was adopted for the conjugation of lectins to the nanoparticles; first step involves the activation of surface associated hydroxyl groups of the PVA of nanoparticles while in the second step glutaraldehyde was used to conjugate lectin to the activated hydroxyl group of nanoparticles (Fig. 3).

Preparation of nanoparticles by emulsification method requires the use of a stabilizer, such as polyvinyl alcohol that becomes incorporated onto nanoparticles surface (Scholes et al., 1999). Polymer may provide a firm anchorage with the PVA on entanglement of polymeric chains at the surface or sub-surface of the matrix resulting into a core-shell structure (Boury et al., 1997). Amount of PVA associated to PLGA nanoparticles was determined by method reported by Joshi et al. (1979). Effect of washing by ultrafiltration on the PVA content and mean diameter of the nanoparticles was assessed. The results indicated that the PVA content and mean diameter did not change significantly ($P < 0.05$) on repeated washing (Table 2). A fraction of PVA remains associated with nanoparticles despite of several washing because PVA forms an interconnected network with the PLGA at the interface. Thus it is inferred that PVA forms a strong adsorbed layer over nanoparticles. This finding is consistent with the report of Murakami et al. (1999) and Cariro et al. (1991). Glutaraldehyde however helps to create and stabilize the surface of PLGA nanoparticles finally providing a strongly adsorbed layer of PVA. The reaction of glutaraldehyde with two adjacent hydroxyl groups in acidic medium leads to the formation of an acetal (Araujo et al., 1997). The free remaining aldehyde groups on the other hand allow either the propaga-

Table 1
Characteristics of lectinized and plain PLGA nanoparticles loaded with HBsAg

Nanoparticles	Average diameter (nm)	Antigen loading (%)	Lectin associated to naoparticles (μg/mg)	Coupling efficiency (%)
Plain NPs	390.72 ± 20.38	54.51 ± 4.80	–	–
PNA-NPs	399.54 ± 21.29	52.18 ± 4.73	17.90 ± 1.14	21.15 ± 0.91

Data represent mean ± S.D. ($n = 4$).

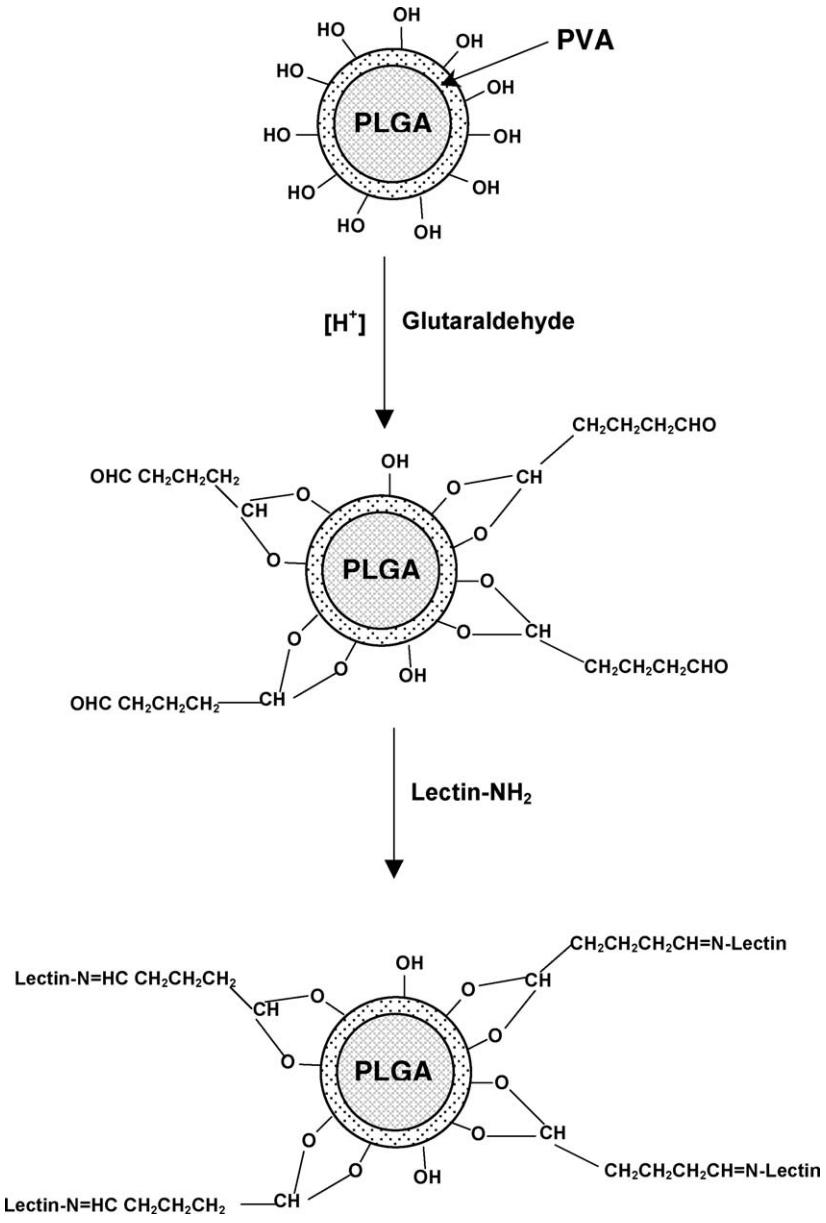


Fig. 3. Schematic presentation of lectin coupling to surface hydroxyl group of PVA via glutaraldehyde.

tion of the cross-linking reaction or the fixation of exogenous molecule e.g. lectins (Montisci et al., 2001).

Lectin was coupled to the PVA associated to the surface of nanoparticles by established method reported by Montisci et al. (2001) with slight modifications. The mixture was incubated for short period of time (1 h) to activate the hydroxyl group. A longer time lead to aggregation (Montisci et al., 2001). The traces of glutaraldehyde, which might otherwise cross-link the lectin molecule, was removed by extensive washings procedure. The concentration of glutaraldehyde in the reaction medium generally varies from 0.20 to 1% (Avrameas, 1969; Ford et al., 1978; Jeanson et al., 1988). We have used higher concentration of glutaraldehyde as reported by Montisci et al. (2001). The use of glutaraldehyde in high concentration would limit the chance of presence of same molecule between hydroxyl group of surface associated PVA of the nanoparticles, this in turn, reduces the possibility of cross-linking. In addition, a two-step method that has been used may limit somewhat polymerization reaction (Hermanson, 1996). Thus the strength of glutaraldehyde used and the set of experimental conditions (brief incubation period, extensive washing procedure and a two step method) ruled out any possibility of cross-linking reaction.

The surface morphology of HBsAg loaded PLGA nanoparticles was investigated by using SEM. As shown in Fig. 2 no major differences could be detected between plain and lectin-coupled nanoparticles. Protein entrapment and anchoring with the lectin do not affect the spherical shape and surface visible texture of nanoparticles. Upon grafting of PNA to the nanoparticles, the mean diameter of the particles however increased marginally (Table 1). This may be attributed to the immobilization of the lectin on the surface of the nanoparticles. Plain nanoparticles showed percentage antigen $54.51 \pm 4.80\%$ loading. The percent-

age antigen loading was recorded to be slightly low in the case of lectinized nanoparticles. The decrease could be attributed to the release of antigen from nanoparticles on incubation employed for anchoring of lectin to the surface of the nanoparticles. Activated nanoparticles were used for the grafting of lectin. The amount of PNA coupled to the nanoparticles was estimated to be 17.90 ± 1.14 μg lectin/mg nanoparticles, which amounts to a coupling efficiency of $21.15 \pm 0.91\%$.

Acidity commonly develops in PLGA formulations due to accumulation of acidic degradation products upon polyester hydrolysis (Shenderova et al., 1999; Brunner et al., 1999). The acidic microclimate in PLGA delivery systems is a potential source of instability of encapsulated proteins. Thus the pH is the potential cause of irreversible inactivation of encapsulated proteins. Peptide bond hydrolysis is particularly fast at acidic pH (Heller, 1990; Zhu et al., 2000; Perez et al., 2002). A rational approach to deter the pH alteration was by inclusion of basic additive in the formulations. A basic salt $Mg(OH)_2$ was thus incorporated at different concentrations into the nanoparticles to lend them retain the structure and biological activity of encapsulated proteins. To assess the neutralization of the acidic environment by $Mg(OH)_2$, the percentage aggregation, PLGA degradation and pH of the release medium were examined. The effects of increasing concentrations of $Mg(OH)_2$ (0.5, 1.0, 1.5 and 2.0%, w/v) on percentage aggregation, PLGA degradation and pH of the medium of each formulation were measured. The higher concentrations of $Mg(OH)_2$ (2.0%) showed significant differences ($P < 0.05$) in percentage aggregation, PLGA degradation and pH of the medium compared to the formulation without $Mg(OH)_2$. The incorporation of $Mg(OH)_2$ virtually eliminated the aggregation (from $62 \pm 5\%$ in absence of base to $2.5 \pm 0.7\%$ on incorporation of base at concentration 2% (w/v)). The degradation half-time was extended from 13 to 28 days, and the pH of the release medium dropped from 7.4 to 6.8 (pH 3.0 was observed without $Mg(OH)_2$; Table 3). On incubation in PBS at 37 °C for 4 weeks the particle size of the plain nanoparticles (pH 3.0) and nanoparticles stabilized 2% $Mg(OH)_2$ (pH 6.8) showed particle size of 407 ± 21 and 437 ± 24 nm, respectively.

3.4. Stability of surface modified nanoparticles

To investigate the linkage between PLGA nanoparticles and lectin, fluorescent labeled lectin (f-PNA) conjugated nanoparticles were incubated in HEPES buffer at 4 °C. During incubation period of 20 days, 9.6% of the total amount of lectin was delodged from activated nanoparticles, on treatment of these nanoparticles with 5 M urea, which is known to disrupt non-covalent interactions, additional 6.5% of the f-PNA was released from the nanoparticles. Nevertheless, approximately 84% of the lectin was estimated to be retained/bound on activated PLGA-nanoparticles.

3.5. In vitro release of HBsAg

To evaluate the suitability of nanoparticles a delivery system for antigen(s), an in vitro release study was conducted with lectinized and plain nanoparticles as well as with protein stabilized nanoparticles. Proteins may unfold and aggregate at the o/w interface therefore one straightforward strategy toward stabilization is to minimize exposure to this interface. Among the various strategies to prevent interface-induced protein denaturation and aggregation, the addition of polyols or sugars excipients in the aqueous phase is well documented (Cleland and Jones, 1996; Perez and Griebenow, 2001). In the present investigation an attempt has been made to stabilize the protein during both, the encapsulation process and the release of protein from the nanoparticles by co-encapsulating a protein stabilizer i.e. trehalose. In this case the protein stabilizer (trehalose) could prevent the antigens from the organic solvent exposure via preferential hydration of the surface.

The HBsAg release pattern from lectin anchored PLGA nanoparticles was studied with different concentrations (0, 0.5, 1.0, 1.5 and 2.0%, w/v) of trehalose (Fig. 4). The release pattern were noted to be typically biphasic with an initial burst release attributed to the release of surface associated protein, followed by a slower release phase which may be accounted for entrapped protein slow diffusion into the release medium (Coombes et al., 1998). In case of PLGA nanoparticles without trehalose 19.5% of the loaded HBsAg was released on day 20 and thereafter the release pattern was consistently constant. This could be due to the antigen inactivation or aggregation at w/o interface (Perez et al., 2002). The deactivated antigen was estimated to be $62 \pm 5\%$ after 2 weeks in case of plain nanoparticles encapsulating HBsAg without protein stabilizer (Table 3). The trehalose reduces the protein denaturation because of its preferential accumulation at the aqueous/organic interface. The

Table 3
Neutralization effect of $Mg(OH)_2$ on the behavior of PLGA nanoparticles loaded with HbsAg

Properties	No salt	Concentration of $Mg(OH)_2$ (%, w/v)			
		0.5	1	1.5	2
Non-covalent aggregates (%)[a]	62 ± 5	46 ± 3	31 ± 3	13.5 ± 2	2.5 ± 0.7
PLGA degradation $t_{1/2}$ (days)[b]	13	15	20	23	28
pH of the medium[c]	3.0	4.0	5.2	6.2	6.8

[a] HBsAg was extracted from nanoparticles after incubation in phosphate-buffered saline (PBS; pH 7.4) at 37 °C for 2 weeks ($n = 4$).
[b] $t_{1/2}$ is the time when the PLGA Mr (determined by GPC; gel permeation chromatography) was reduced to half of the original Mr during incubation in PBS at 37 °C ($n = 4$).
[c] PBS medium containing 5 mg polymer nanoparticles after incubation at 37 °C for 4 weeks ($n = 4$).

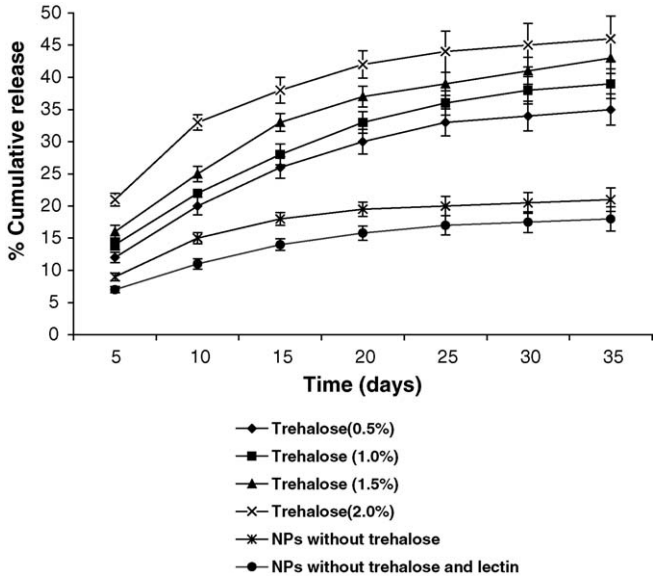


Fig. 4. In vitro cumulative release of HBsAg from various PNA coupled PLGA nanoparticles (stabilized with 0.5, 1.0, 1.5 and 2.0% trehalose) and plain PLGA nanoparticle formulation ($n = 4$).

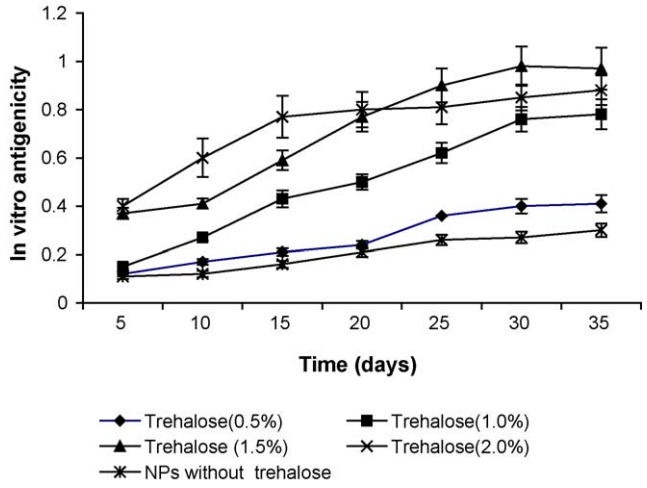


Fig. 5. In vitro antigenicity (response of enzyme immunoassay to protein concentration) of HBsAg in lectin-coupled PLGA nanoparticle (with trehalose concentration of 0, 0.5, 1.0, 1.5, 2.0%) during in vitro release study.

trehalose being hydrophilic in nature dissolves rapidly from the polymeric sheath leaving porous matrix and as a result the formulations containing trehalose showed increased release profile in comparison to the formulation without trehalose. As concentration of trehalose increased the release profile also increased, however higher concentration (2%) resulted in higher initial burst of antigen, whereas at 1.5% trehalose concentration the initial burst was 16% on day 5 followed by a sustained release of antigen up to day 35. The higher burst of lectin coated as compared to uncoated nanoparticles may be due to the hydrophilic characteristics of lectin, allowing easier penetration of aqueous solution into the matrix thereby dissolving the protein (Walter et al., 2004).

The incomplete protein release was observed during the investigated time span, which is the commonly encountered problem in protein–PLGA formulations (Aubert-Pouessel et al., 2002). Incomplete release may be attributed to non-covalent aggregation and hydrophobic PLGA–protein interaction. Incomplete release kinetic profiles are dependent on polymer degradation-associated protein instability events such as protein aggregation and protein adsorption onto the enlarging surface area of degrading nanoparticles/microparticles. The possibility of incomplete release cannot be ruled out by ionic interactions between positively charged amino acid residue in encapsulated protein molecule and carboxylic group in the PLGA oligomer generated from scissioned polymer backbone (Park et al., 1998). In addition, the residual PVA associated with nanoparticles may also affect release profile. The smaller size of nanoparticles presents a large surface area for the protein to diffuse out more rapidly compared to that from microparticles. However, nanoparticles contain more surface associated PVA, which could present a significant barrier to the outward diffusion of the encapsulated protein (Panyam et al., 2003). The higher viscosity of the 5% PVA solution which was used in manufacturing process, could have resulted in a more compact polymer matrix resulting in lowered degradation rate of the polymer and/or the slower diffusion of the encapsulated antigen from nanoparticles. The residual PVA does not affect the amount of protein associated with surface of the nanoparticles but possibly influences the diffusion and degradation of the polymer matrix (Sahoo et al., 2002). PVA is a swellable, hydrophilic macromolecule that has previously been shown to sustain the release of macromolecules (Kushwaha et al., 1998). PVA could swell in water and thereby reduce the availability of water molecule for PLGA degradation. Thus surface associated PVA could form a hydrogel barrier to the diffusional release of macromolecule encapsulated in nanoparticles. All these factors contribute to a significant amount of HBsAg remained in the nanoparticles during in vitro release study.

Antigen should maintain their antigenicity during in vitro release therefore lectin anchored HBsAg loaded nanoparticles were stabilized using trehalose and evaluated for antigenicity during in vitro release. The in vitro antigenicity of the antigen was evaluated in terms of EIA/protein ratio. Fig. 5 depicts the in vitro antigenicity of HBsAg stabilized with different concentration of trehalose. Lectin-coupled PLGA nanoparticles revealed EIA/protein ratio 0.41, 0.78, 0.97 and 0.88 at trehalose concentration of 0.5, 1.0, 1.5 and 2.0%, respectively. Lectin-coupled PLGA nanoparticles with trehalose concentration 1, 1.5 and 2% showed significantly higher in vitro antigenicity as compared to PLGA nanoparticles without trehalose. The antigenicity of HBsAg in lectin-coupled PLGA nanoparticles without trehalose relatively low owing to the denaturation of antigen as the released antigen was exposed to in vitro release medium at 37 °C up to 35 days. Release profile and in vitro antigenicity study showed that 1.5% trehalose concentration resulted in better-controlled release as well as maintained the immunogenicity of HBsAg. The results are in accordance with our previous report (Jaganathan et al., 2004).

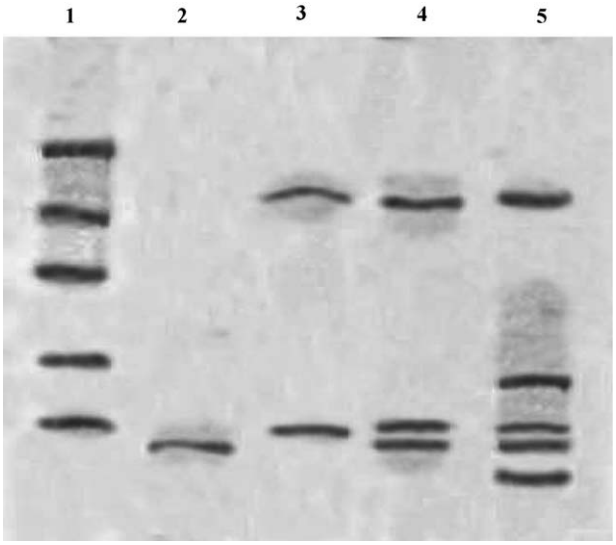


Fig. 6. SDS-PAGE analysis of lectin anchored nanoparticles bearing HBsAg. Lane 1: molecular weight marker; marker proteins; 205 kDa myosin, rabbit muscle; 97.4 kDa phosphorylase b; 66 kDa bovine serum albumin; 43 kDa ovalbumin; 29 kDa carbonic anhydrase. Lane 2: native HBsAg. Lane 3: native PNA. Lane 4: HBsAg loaded lectin anchored nanoparticles stabilized with trehalose. Lane 5: HBsAg loaded lectin anchored nanoparticles without stabilization by trehalose.

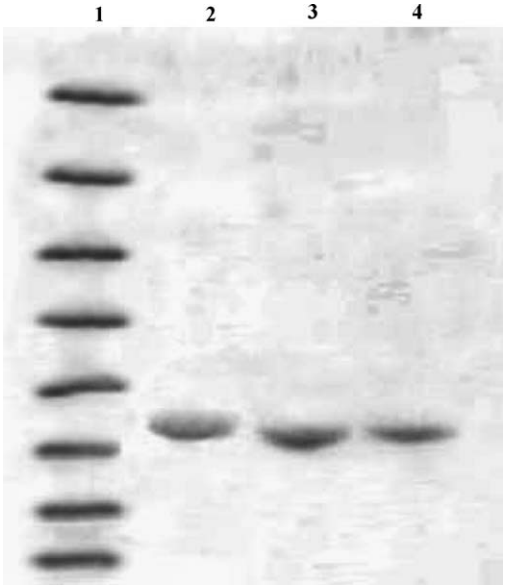


Fig. 7. Isoelectric focusing of HBsAg—lane 1: standard marker proteins; lane 2: native HBsAg; lane 3: HBsAg loaded nanoparticles stabilized with trehalose; lane 4: alum adsorbed HBsAg.

3.6. Structural integrity of HBsAg

In order to assess the structural integrity of antigen encapsulated in the lectin-coupled PLGA nanoparticles, SDS-PAGE measurement was employed. It can be seen from Fig. 6 that the native HBsAg and HBsAg extracted from lectin-coupled PLGA nanoparticles stabilized with trehalose showed single band of same molecular weight (MW 24 kDa). It is suggested that no chemical polymerization, non-covalent aggregation or molecular hydrolysis occurred during the preparation process. However, the integrity of the HBsAg without trehalose got altered following encapsulation in PLGA nanoparticles due to protein unfolding and aggregation at o/w interface.

Isoelectric focusing is an ideal method for the separation of amphoteric substances such as protein because it is based on the separation of molecule according to their different isoelectric points. Further this is one of the most suitable methods to test the most common route of protein degradation i.e. deamidation of encapsulated and released antigen (Zhu et al., 2000). The isoelectric focusing analysis (Fig. 7) have revealed that there was no change in the isoelectric point of HBsAg (5.2–5.5) in case of native HBsAg, alum adsorbed HBsAg and encapsulated HBsAg when stabilized with trehalose.

3.7. In vitro ligand affinity and activity studies

Due to the presence of numerous functional groups (i.e. amino and carboxylic residues) the proteins are excellent candidates for the preparation of conjugates, through attachment of ligand capable of providing specificity to the surface of nanoparticles such as lectins. In the present investigation BSM (bovine submaxillary mucin), a glycoprotein, was used as a biological model to determine the in vitro activity and specificity of PNA coupled nanoparticles towards sugar residue of glycoprotein. The carbohydrate part of the BSM is composed of six sugars; *N*-acetylgalactosamine, *N*-acetylglucosamine, galactose, mannose, fucose and sialic acid (Honda and Suzuki, 1984; Vyas et al., 2001b). For the determination of in vitro activity of PNA, experiment was carried out in the absence of specific sugar and for the determination of in vitro specificity of PNA, the experiment was carried out in the presence of the specific sugar (D-galactose) for PNA. In the absence of D-galactose, the PNA-nanoparticles exhibited almost four times higher interaction with BSM than unmodified nanoparticles (Fig. 8). In the presence of D-galactose the interaction between PNA coupled nanoparticles and BSM was low. Plain nanoparticles revealed fairly comparable ($P < 0.05$) results in absence and in presence of specific sugar for PNA. The interaction between lectinized nanoparticles and BSM was significantly ($P < 0.05$) decreased in the presence of D-galactose. Thus results suggest that lectinized nanoparticles

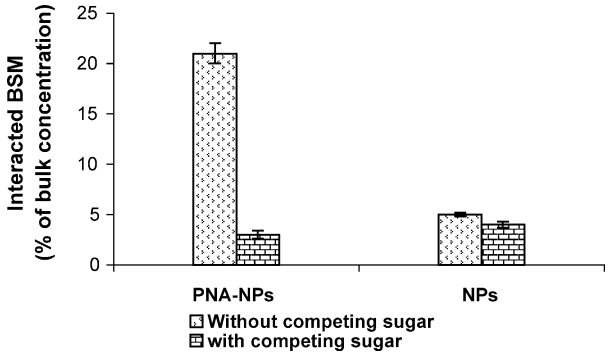


Fig. 8. Binding of BSM to PNA coupled PLGA nanoparticles (PNA-NP) and plain PLGA nanoparticles (NP) in suspension with and without competing sugar (D-galactose at concentration of 100 mM) ($n = 4$).

retain activity and same sugar specificity as the native lectin PNA.

4. Conclusion

Co-encapsulation of protein stabilizer, trehalose and antacid $Mg(OH)_2$ is the potential approach towards the stabilization of HBsAg encapsulated in PLGA nanoparticles. The antigen retains its integrity in the presence of trehalose as evidenced by SDS-PAGE analysis and isoelectric focusing. The ligand-coupled nanoparticles were found to retain ligand-binding activity of surface anchored PNA as demonstrated by bovine submaxillary mucin binding assay. Thus these novel ligand-coupled PLGA nanoparticles would be potential antigen carrier for the oral-targeted delivery of antigen to antigen presenting cells of the Peyer's patches. The interaction of these lectinized nanoparticles with the Peyer's patch cells and the in vivo immune response against Hepatitis B antigen will be reported and discussed in a later paper.

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