M-cell targeting acid-resistant oral vaccine delivery for immunization against Hepatitis B infection using cationic solid lipid nanoparticles

Surendra Saraf[1], Rudra Narayan Sahoo[2], Subrata Mallick[3]

1 Columbia College of Pharmacy Raipur
2 Centurion University of Technology and Management
3 Siksha 'O' Anusandhan University

Funding: The author(s) received no specific funding for this work.

Potential competing interests: The author(s) declared that no potential competing interests exist.

Abstract

Purpose: Viral infection caused by Hepatitis B is transmitted by permucosal or parenteral exposure and also one of the prime causes of hepatocellular carcinoma and liver cirrhosis.M-cell targeting acid-resistant oral vaccine delivery have been formulated for immunization against Hepatitis B infection.

Method: Cationic solid lipid nanoparticles (cSLNs) were prepared utilizing solvent injection technique. Hepatitis B surface antigen (HBsAg) loaded alginate coated cSLNs were anchored with lipopolysaccharide (LPS). SDS-PAGE was performed to evaluate acid degradation protection of prepared formulation.

Result: Induction of immunity produced by prepared nanoparticle for Hepatitis B was determined on female Balb/c mice followed by ELISA assays for assessing anti-HBsAg IgG/IgA antibodies in mucosal fluids.Sustained release of HBsAg (60.66%) has been exhibited from alginate coated cSLNs in comparison to cSLNs without alginate coating (97.72%) after 48 *h*. The production of anti-HBs titer in intestinal, salivary and vaginal secretions was 3.41 IU/ml, 3.1 IU/ml and 2.51 IU/ml respectively in comparison to the control group.

Conclusion: Integrity of the M-cells has been maintained after binding with SLN, and oral administration delivered the antigen to the desired site of gut. It was found effective in producing antibodies in mucosal immunization against Hepatitis B virus. So, this formulation could be used as a promising alternative preexisting vaccine to prevent Hepatitis B infection.

Surendra Saraf[1], **Rudra Narayan Sahoo**[2], **Subrata Mallick**[3*]

[1] *Columbia College of Pharmacy Raipur, Chhattisgarh, India.*

[2] *School of Pharmacy and Life Sciences, Centurion University of Technology and Management, Odisha, India.*

[3] *School of Pharmaceutical Sciences, Siksha'O'Anusandhan (Deemed to be University), Bhubaneswar-751003, Orissa, India.*

Running Title: Promising vaccine to prevent Hepatitis B infection

Keywords: Hepatitis B surface antigen; Oral vaccine delivery; LPS Anchoring; MTT assay; Cationic solid lipid nanoparticles.

Introduction

Hepatitis B, a viral disease is transmitted by permucosal or parenteral exposure and the cause of nearly 4 million acute infections worldwide as per one recent report[1]. Hepatitis B is also one of the prime causes of hepatocellular carcinoma and liver cirrhosis[2]. The host immune system plays very crucial role in the prevention of viral infection and along with reduction of inflammation occurs in liver due to Hepatitis B infection[3].Thus, recovery from Hepatitis B virus infection is dependent on adaptive immune response of the host[4]. In this concern, vaccination has been gaining much importance for producing immunity against hepatitis B virus (HBV) infection for maintaining long lasting immunologic memory to protect an individual from subsequent diseases[5]. Vaccines given through parenteral routes are associated with several disadvantages such as pain, less patient compliance, high cost and poor immunogenicity because of producing only systemic immunity[6].To overcome these limitations oral mucosal immunization has attracted much attention[7]. Non-invasive technique, immunity against selective pathogen and induction of both local and systemic immunity are the several advantages of oral mucosal immunization through production of antibodies i.e. IgA and IgG, respectively[8]. It is one of the most accepted route for delivery of antigens safely almost without undesired toxicity; while, maintaining the integrity of antigen is very much challenging[9].

To overcome the associated problem with the delivery of antigen through oral mucosal route, nanocarriers are very much convenient and advantageous in the production of vaccines and delivery of antigens to stimulate desired immune response. This delivery system is known for controlling release, specific targeting, antigen protecting and uniformly distributing into the systemic circulation. The immunogenicity of these vaccines can be enhanced by incorporating with the adjuvants[10].Adjuvants act as immune-simulators in the formulation of vaccines[11].The conventional adjuvants (e.g. Incomplete Freund, Chitosan nanoparticles and Aluminum hydroxide adjuvants) have been replaced with outer membrane vesicles (OMVs). These vesicles have tremendous utility as adjuvants due to stimulation of innate and adaptive immunity. In addition, vesicles have low toxicity and used as advanced mucosal antigen delivery carrier[12]. Adjuvants induce antigen presentation and activate antigen-presenting cells for long-lasting memory of immunity in the body. Lipids are also good adjuvants and lipid derived nanoparticles have been proved effective for antigen delivery to induce mucosal immunization[5].

Cationic solid lipid nanoparticles (cSLNs) are the colloidal carriers that are made up of biocompatible lipids. It contains at least one cationic lipid that acts as a vector for antigen delivery[13]. The size of cSLNs ranges from 10 to 1000 nm extensively used for oral delivery of antigens to induce immunity[14]. Cationic solid lipid nanoparticles (cSLNs) have wide application in antigendelivery, drugs and other phytoconstituents in the treatment of various diseases. It is a preferred delivery system due to certain advantages like more stability, bioavailability, biodegradability, nano size range of

particles, low toxicity, applicable to both hydrophilic and lipophilic drugs, no leaching problem and increased uptake of antigens by lymphatic through oral delivery of vaccines[15]. cSLNs is the most promising delivery system for antigens that overcome the limitations of polymeric nanoparticles and other lipid based nanoparticles. cSLNs are made up of biocompatible lipids that allows better compatibility and low cytotoxicity[16].

However, cSLNs must be target specific to bind with M cell located at mucosa. cSLNs are the suitable adjuvants for delivery of antigens to be used as a cocktail vaccine with considerable low cytotoxicity[17]. cSLNs as the carriers have been approved for delivery of antigens and other pharmaceutical applications[18]. However, proteinous nature of the antigen at the mucosal sites is liable to degradation resulting the treatment ineffective[19]. To accomplish this task adjuvants mimicking certain defined sets of stimulatory molecules like bacterial cell wall, lipopolysaccharides (LPS), endocytosed nucleic acids, etc. are used. Lipopolysaccharides (LPSs) are generally anchored with nanoparticles (NPs) to make them M cell targeting for oral mucosal immunization. LPS present in outer membrane of gram-negative bacteria and prevents it from pathogen attack[20]. Poly(allylamine hydrochloride) (PAH) was coated onto outer surface of vesicles to make them cationic. Thus, the protocol of the study was designed to formulate LPS derived HBsAg-loaded cSLNs for oral mucosal immunization. The formulation was characterized by evaluating particle size, polydispersity, zeta potential, release pattern, loading capacity and immunological response in mice.

Experimental

Materials

Hepatitis B surface antigen (HBsAg) was received as free sample for conducting research. Tristearin (Mol. wt. 891.5 g/mol, Technical grade), stearylamine (Mol. wt. 269.5 g/mol, Technical grade) and lactose monohydrate (Mol. wt. 360.32 g/mol) were purchased from Himedia, India. Sodium alginate (Mol. wt. 85,000-290000, Grade III), Lipopolysaccharide (Mol. wt. 50000-100000) and Antibodies (IgG and IgA) were procured from Sigma Aldrich, USA. Protein assay kit (Bicinchoninic acid) was obtained from Genei, India. All other chemical and reagents were of analytical grade or higher.

Method of cSLNs preparation

Solvent injection method was used for preparation of cSLNs with slight modification[5]. In this method, tristearin (2%) and stearylamine (0.5%) was dissolved in acetone (2.5 ml) at room temperature to form organic phase. Lactose monohydrate (2.5%) was added in aqueous solution containing Tween-80 (0.5%). Then organic phase was rapidly injected into the aqueous phase with continuous stirring through injection needle. It results into dispersion which was filtered through whatman filter paper and it was lyophilized (Multitech Scientific Instruments Pvt. Ltd, Raipur, India). Fig. 1A shows the graphical representation of preparation of solid lipid nanoparticles.

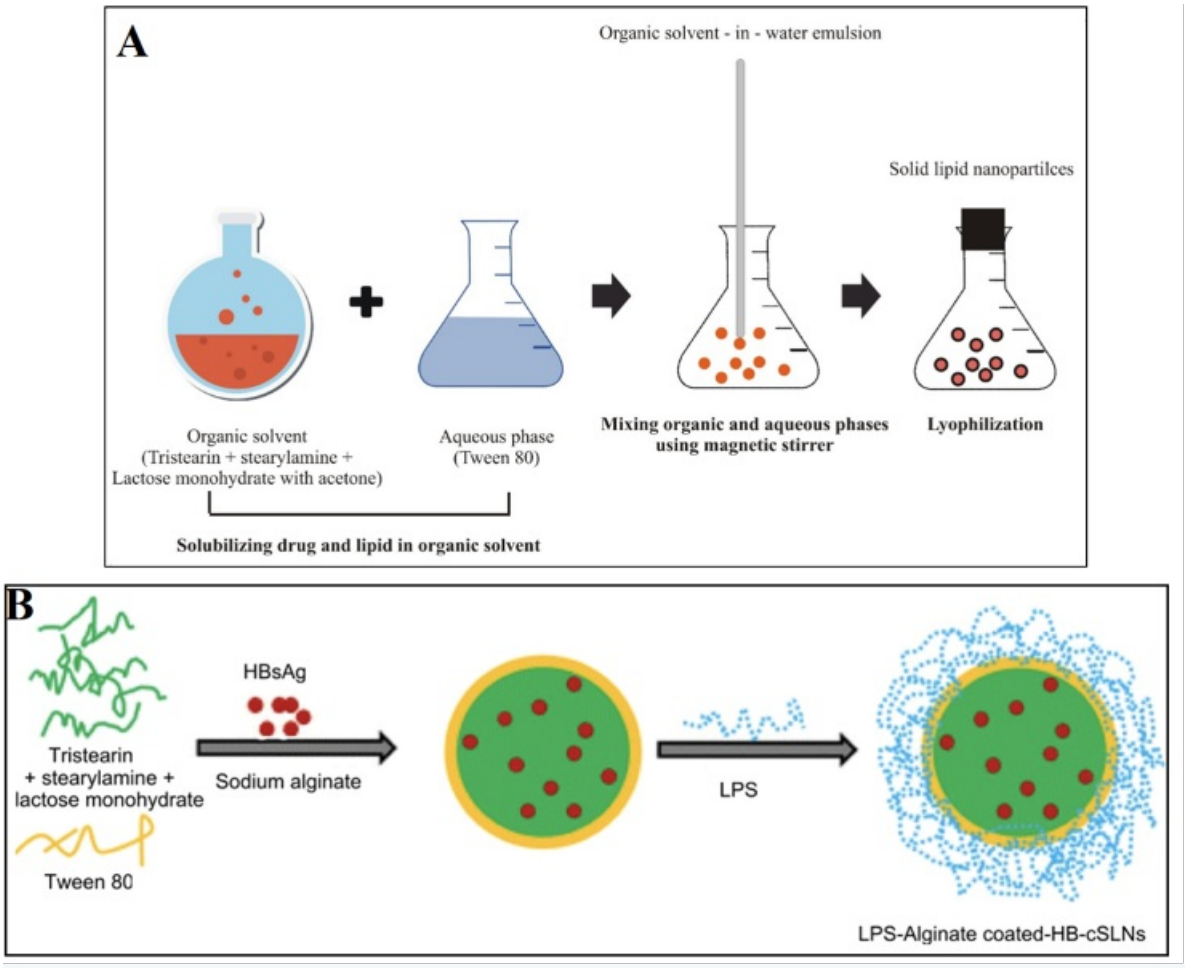

Figure 1. *(A) Flow chart for preparation of solid lipid nanoparticles, (B) Schematic representation of LPS anchoring with nanoparticles*

Preparation of HBsAg-loaded alginate coated cSLNs

Lyophilized NPs of cSLNs were re-dispersed in distilled water (25 ml) at concentration of 5 mg/mL under continuous ultrasonication (Model PCI 750F, PCi analytics, India) to disaggregate the NPs. Different concentrations of HBsAg (0.5%, w/v) in aq. phase was incubated with prepared cSLNs under agitation for 120 min at room temperature to allow adsorption of HBsAg over the surface of cSLNs. In the next step, coating sodium alginate was performed over these HBsAg adsorbed cSLNs. For this equal volume of cSLNs suspensions were mixed with sodium alginate solution (1%, w/v) using magnetic stirrer upto 20 min. Then it was centrifuge for 10 min at 16000 rpm and supernatant was discarded. After that precipitate was re dispersed into solution of calcium chloride ($CaCl_2$, 0.524 mM) under mild agitation for 10 min for cross-linking of alginate present on the surface of cSLNs. So alginate coated HBsAg adsorbed cSLNs was prepared for further linkage with LPS[21].

LPS Anchoring with HB-cSLNs

LPS was anchored on HB-cSLNs using layer-by-layer (LbL) technique to form LPS anchored alginate coated HBsAg loaded cSLNs (LPS-Alginate coated-HB-cSLNs)[22]. PAH get coated on LPS-Alginate coated-HB-cSLNs as an intermediate linker to give cationic charge to cSLNs. The additional coating of negatively charged material improved stability of prepared cSLNs. LPS coating on the outside of PAH-cSLNs was done due to interaction between LPS (PO_3^-) and PAH (NH_3^+). Fig. 1B represents the scheme for LPS anchoring with HB-cSLNs.

Evaluation of cSLNs loaded with HBsAg

Particle size and morphology

The size of nanoparticles and their internal structure were assessed using transmission electron microscopy (TEM) [TECNAI 200 Kv]. In this method, the samples were stained using 2% phosphotungstic acid. This analysis allows to assess capacity of NPs for its anchoring with LPS and HBsAg entrapment.

Particle size, polydispersity and zeta potential

Photon correlation spectroscopy (PCS) was used for determination of size of particle along with polydispersity. It was measured using Malvern Nano ZS zetasizer. Laser Doppler Anemometry was used for determination of Zeta potential. The dispersion samples of cSLNs were 1:20 diluted with MilliQ water before analysis. The measurements were performed in triplicate.

Entrapment efficiency of HB-cSLNs-LPS

Entrapment efficiency is described in percentage as the drug successfully entrapped in the nanoparticle. It is one of the most important physiochemical characteristics of cSLNs.For determining loading efficiency, 20 mg of the nanoparticles was dissolved in Triton X-100 (w/v) in the concentration of 0.1% of volume 2 ml in 0.1 M PBS. BCA colorimetric assay was used to determine antigen amount. The difference between protein content of LPS incorporated HBsAg loaded cSLNs and LPS incorporated blank nanoparticle is the amount of HBsAg in LPS decorated cSLNs[23]. The entrapment efficiency (EE) values were calculated according to the following equations:

$$EE(\%) = [(\text{Total amount of HBsAg} - \text{Free HBsAg}) / \text{Total amount of HBsAg}] \times 100$$

2.6 Antigen release study

In vitro release of antigen (HBsAg) and of chitosan nanoparticles (alginate coated) was studied in simulated gastric fluid (0.1M HCl) for 2 h followed by simulated intestinal fluid (PBS, pH 7.4) for 2 h followed by incubation 37 ± 1°Cfor 5-6 h. The solution aliquots were removed by vortexing the eppendorf tubes at predetermined time intervals. The solution was centrifuged at 11,000 rpm for 5 min and supernatant was used to analyze BCA protein[24]. The major hurdle for oral delivery of drug is the varying conditions of gastrointestinal tract especially physiological fluid pH. The pH at different sites

of GIT are 1.0 to 2.5 at stomach, 6.6 to 7.5 at proximal end to the ileum of the small intestine, 6.4 at the ceacum, and 7.0 at the left colon[25].

SDS PAGE analysis for HBsAg integrity testing

The analysis was carried out for ascertaining antigen stability entrapment in the formulation. In this technique, the separation was based on the size of the protein. SDS, an anionic detergent was loaded into polyacrylamide gel and the electric field has been applied. This leads to the separation of SDS-coated proteins. The SDS coated proteins proceeded towards the anode due to acting electric field in which small protein moved faster towards anode rather than large proteins. The standard proteins are loaded with SDS along with test proteins in order to identify size of proteins. Briefly, cationic nanoparticles were centrifuged at 13000 rpm followed by removing supernatant after 20 min. It was then incubated with HCl (0.5 ml, 0.01 M) and allowed to stand for 2 h at 37 °C. Aq. NaOH was used to stop reaction and allowed to sustain release for 24 h by adding PBS to final volume. HBsAg was analyzed by SDS-PAGE after 24 h[26].

Characterization of mucoadhesion test

The mucoadhesion test was carried out by dipping cellulose nitrate membrane with pore size of 0.45 µm in 0.1% of mucin solution and then mucoadhesion of LPS-HB-cSLNs to this membrane was determined by applying drop-wise to the core of the membrane. It was washed with PBS followed by drying in vaccum. The difference in weight of soaked membrane and membrane loaded with nanoparticle was calculated as the mucoadhesion of nanoparticles. The mucoadhesion study was performed in triplicate[27].

Stability studies

Stability studies of cSLNs were performed upto 60 days to validate the quality of formulation that might have changed if any with the time due to different environmental factors such as light, humidity and temperature. Blank nanoparticles (NPs) and cSLNs were stored at two different temperature conditions i.e. 4±1°C and 27±2°C. It was followed by observing at different time interval i.e. 10, 20, 30, 45 and 60 days for any change in size of particle and protein content[28]. The nanoparticles have been exposed to 4 +/- 1 and 27 +/- 2 °C followed by determination of protein content using BCA™ kit. The initial protein content was considered as 100% for each formulation and then percentage residual protein content was calculated.

Evaluation of cytotoxicity by MTT Assay

Assay was performed by seeding confluent cells into 96 well culture plates for 24 h. Each cell containing 100 µl of antigen along with LPS-HB-cSLNs in various concentrations (12.5, 25, 50, 100, 200, 400, 800, and 1600 µg/ml) for 24 and

48 h followed by addition of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) dye in each well and incubated at 37 C for 4 h. The viable cells reduced MTT into formazan crystals. The optical density of formazan crystals was estimated at 562 nm[29]. Percentage viability of cell has been evaluated using following formula:

$$\text{Percent viability of cell} = (\text{Sample OD} / \text{Control OD}) \times 100$$

Induction of immunity

Induction of immunity produced by prepared nanoparticle for hepatitis B was determined on female Balb/c mice. It is an approved study by IAEC, Columbia Institute of Pharmacy, Raipur (Regd. No- 1321/PO/ReBi/S/10 CPCSEA). Animals have full access to food and water except whole night prior to oral immunization. Seven groups of animals were prepared each containing six animals. Group I was Control group received PBS; Group II was Blank cSLNs group containing 10 µg dose of cSLNs; Group III was Blank HB group received 10 µg dose of HBsAg; Group IV was HB-cSLNs group received 10 µg dose of HBsAg loaded cSLNs, Group V was HB-A cSLNs group containing 10 µg dose of nanoparticle loaded with HBsAg and coated with alginate; Group VI was LPS-HB-A cSLNs containing 10 µg/0.5 mL dose of HBsAg-loaded alginate cSLNs (LPS anchored); Group VII was HB-A group containing 10 µg dose of Alum-HBsAg given intramuscularly. However, all other treatment were given per oral to different groups of animals. The pre-immunization serum collection was done on day 0. Blood was collected periodically in every two weeks post-immunization through retro-orbital route of mice. The collected serum was stored at -40°C till further use. However, other secretions were collected post-immunization (after 5 weeks); these fluids were vaginal, salivary and intestinal secretions. It was stored with phenylmethyl sulfonyl fluoride (PMSF, 100 mM) for examination of secretory antibody (sIgA and sIgG) levels using ELISA at -40 °C.

Collection of blood and fluids

The Blood sample was collected at 0, 14, 28, 42 and finally 56 days through the retro-orbital route of mice. The collected serum was separated from the collected blood samples after centrifugation which was stored at -40 °Ctill further use. However, other secretions were collected post-immunization (after 5 weeks); from the vaginal, salivary and intestinal secretions and it was stored with phenylmethyl sulfonyl fluoride (PMSF, 100 mM) for examination of secretory antibody (sIgA) levels using ELISA at -40°C.

Saliva of immunized animals were collected by intraperitoneal injecting pilocarpine solution at the dose of 10 mg/ml. After 30 minutes of this injection animals started salivation which was collected by using capillary tube and transferred it into ependroff tube. For collecting vaginal fluid, vaginal administration was done using phosphate buffer saline (pH 7.4) with BSA (1 %). Intestinal lavage (20 mM of NaHCO3, 25 mM of NaCl, 10 mM of KCl, 48.5 mM of PEG and 40 mM of Na2SO4) was administered intragastrically for collecting intestinal secretions at 15 min intervals using blunt ends of feeding needle. After last dose of intestinal lavage solution (30 min), pilocarpine (0.2 ml) was administered (ip) and carefully fluids were collected in a tube.

ELISA test for estimation of serum IgG and mucosal sIgA

ELISA method was performed for qualitative estimation of produced antibodies in serum and mucosal fluids against HB_SAg in different groups of animals. To perform this procedure each well 100 µl of solution placed in wells of the microtiter plate coated with HB_SAg (100 ng equivalent) in 50 mm phosphate buffer solution and plate was incubated for 90 min at 37 ± 1 °C. The plate was washed 3 times using PBS (containing Tween 80, 0.1 %) for removing excess unbound HBsAg and the blocking buffer was added for blocking free site available on the antibodies. Again the plate was incubated at 37 ± 1 °C and washed thrice with PBS (containing Tween 80, 0.1 %). Serum was added (100 µl) in each HBsAg pre-coated wells and 2 hours given for incubation. Each well of micro titre plates was washed with above PBS five times. Horseradish Peroxidase conjugated goat anti-rat IgG antibodies was then added in every well and placed for incubation for 2 hours. As substrate, O-Phenylenediamine dihydrochloride was added (100 µl) and covered with aluminium foil for colour development. For stopping reaction 50 ml of 5N H_2SO_4 was added 10 min after for stopping reaction. ELISA plate reader was used for developed colour measurement at 492 nm (Biotek, Elx 800).
HBsAg coated microtitre plates (2 µg/ml) were incubated at 4 °C for overnight period for determining sIgA in mucosal fluid. All the wells were washed three times with phosphate buffer saline (Tween 80, 0.1 %) and incubated at 37 °C for 2 hours after blocking with 3% w/v BSA in PBS as blocking buffer. Wells of the plates were washed three times with PBS-T and mucosal secretion in diluted state with PBS-BSA (0.1%, w/v) was added in HBsAg coated wells and incubated at 37 °C for 2 hours. Unbound antibodies were removed by using PBS-T. HRP-conjugated goat anti-rat IgA antibodies were added to the wells and incubated 2 h. The substrate OPD in citrate-phosphate buffer (pH 5.0) containing H_2O_2 was added after washing with PBS-T. The colour reaction was measured by ELISA plate readers (Biotek, ELx 800) at 492 nm[30].

Statistics

Data are shown as mean ± SD. Statistics was performed using one way ANOVA for comparing different groups through Graph Pad Prism, statistically different at $p<0.05$.

Results

Preparation of nanoparticles

cSLNs were prepared by mixing tristearin (2%), stearylamine (0.5%) with lactose monohydrate (2.5%) and surfactant tween 80 (0.5%) at room temperature. It was further loaded with HBsAg (0.5%) and coated with sodium alginate (1%) and then derived by LPS as shown in Table 1. It was optimized for several process variables such as surfactant concentration, stirring speed and lipid concentration for polydispersity, particle size and zeta potential. While increasing the lipid content, increase in the particle size and zeta potential was seen. The particles were irregular aggregates when stirring time was 1

h; however, while increasing the stirring time from 1 to 6 h, a homogenous preparation was obtained with optimum particle size, polydispersity and zeta potential. The particle size and polydispersity were decreasing in reciprocating to surfactant up to certain extent.

Table 1 Formulation and characterization of cationic solid lipid nanoparticles (cSLNs)

Formulations	Ingredients							Physiochemical properties		
	Tristearin (%, w/v)	Stearylamine (%)	Lactose monohydrate (%, w/v)	Tween 80 (%, v/v)	HBsAg (%, w/v)	Sodium alginate (%, w/v)	LPS (%)	Mean particles size (nm)	PDI	Zeta potential (mV)
cSLNs	2	0.5	2.5	0.5	–	–	–	207.18±3.22	0.171±0.003	+36.81±0.17
HB- cSLNs-I	2	0.5	2.5	0.5	0.3	–	–	248.16±2.12	0.182±0.002	+35.21±0.32
HB- cSLNs-II	2	0.5	2.5	0.5	0.5	–	–	237.32±3.73	0.169±0.004	+33.44±0.11
HB-AcSLNs-I	2	0.5	2.5	0.5	0.5	1	–	287.36±2.34	0.165±0.002	-31.15±0.23
HB-AcSLNs-II	2	0.5	2.5	0.5	0.5	2	–	345.76±2.71	0.178±0.002	-33.86±0.21
LPS-HB-AcSLNs	2	0.5	2.5	0.5	0.5	1	1	313. 82±2.68	0.162±0.011	-28.24±0.14

Values were determined as mean±SD (n=3). cSLNs = Cationic solid lipid nanoparticles, HB-cSLNs = Hepatitis B antigen loaded cSLNs, HB-AcSLNs = Alginate coated Hepatitis B antigen loaded cSLNs, LPS-HB-AcSLNs = Lipopolysaccharide derived alginate coated Hepatitis B antigen loaded cSLNs.

Characterization of LPS-Alginate coated HB-cSLNs

Table 1shows the characterization of LPS-Alginate coated HB-cSLNs. cSLNs showed the particle size of 207.18 nm with PDI, 0.171 and zeta potential, +36.8 mV. It may be due to the presence of optimized lipid and lecithin ratio with surfactant. This preparation was further subjected to loading of HBsAg onto to cSLNs. It increased the particle size to 237.32 nm along with increase in zeta potential (+33.4 mV) but decreasing polydispersity (0.169). It indicated that the loading of HBsAg on cSLNs leads to more stabilization due to increased zeta potential. The particle size of cSLNs was further raised to 287.36 nm due to coating of alginate and zeta potential raised to -31.1 mV with decrease in polydispersity (0.165). However, LPS-HB-AcSLNs exhibited optimum particle size of 313.82 nm with polydispersity 0.162 and zeta potential of -28.2 mV due to anchoring of 1% LPS. Thus, LPS-Alginate coated HB- cSLNs was used for immunization study.

cSLNs surface morphology

TEM was used to know particle size as well as morphology of cSLNs, results revealed that prepared nanoparticle were spherical in shape and nano sized range (Fig. 2A). The particles with smaller size have more positive attributes like

good stability and conductivity that make nanoparticles suitable for biological applications.

In vitro protein release of LPS-Alginate coated HB-cSLNs

The cumulative release profile of the alginate coated cSLNs exhibited a slow pattern (60.66 %) in comparison to cSLNs without alginate coating showing 97.72 % after 48 h. Sustained release of HBsAg was possible only due to the alginate coating of the nanoparticles effectively. The integrity of HBsAg released from cSLNs was evaluated by SDS-PAGE and is shown in (Fig. 2B).

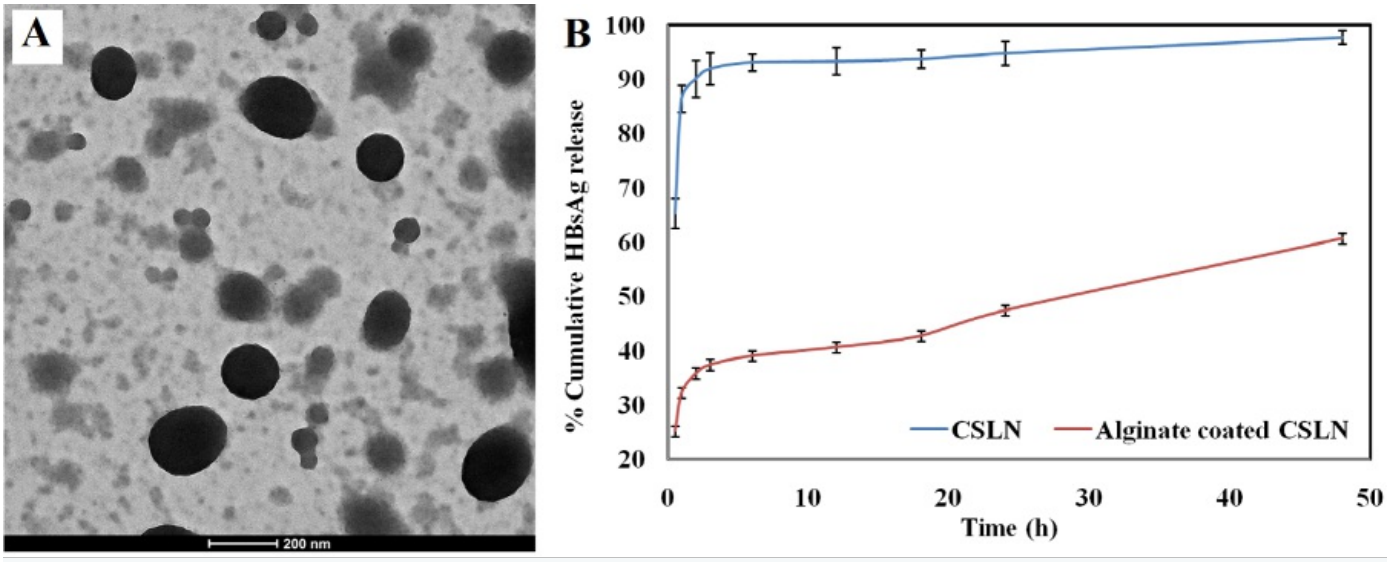

Figure 2. *(A) TEM image of prepared nanoparticles (LPS-HB- AcSLNs); (B) In-vitro release of protein from cationic solid lipid nanoparticles. Data are represented as mean ±SD (n=3).*

Analysis of HBsAg integrity by SDS-PAGE

The bands obtained from SDS-PAGE were observed and confirmed that HBsAg was endured the loading and release processes at 37°C. However, the loaded HBsAg (1 and 2 days old) was not different from that of freshly prepared HBsAg standards. Therefore, it could be concluded that HBsAg remained in its native form in the cSLNs under the experimental conditions. (Fig. 3) exhibits Lane 1 showing molecular weight of markers; Lane 2 defines HBsAg purity at about 24 kD; HBsAg incubated with PBS (pH7.4) was revealed in Lane 3; a faint band observed in Lane 4 (0.01 M HCl pretreated) shows HBsAg 24 KD.

Mucoadhesion study of nanoparticles

Mucoadhesion study revealed that alginate coated cSLNs exhibited good binding as compared to blank cSLNs. The adhesion was decreased after washing in blank nanoparticle, however, cSLNs was resistant to washing for longer period

of time.

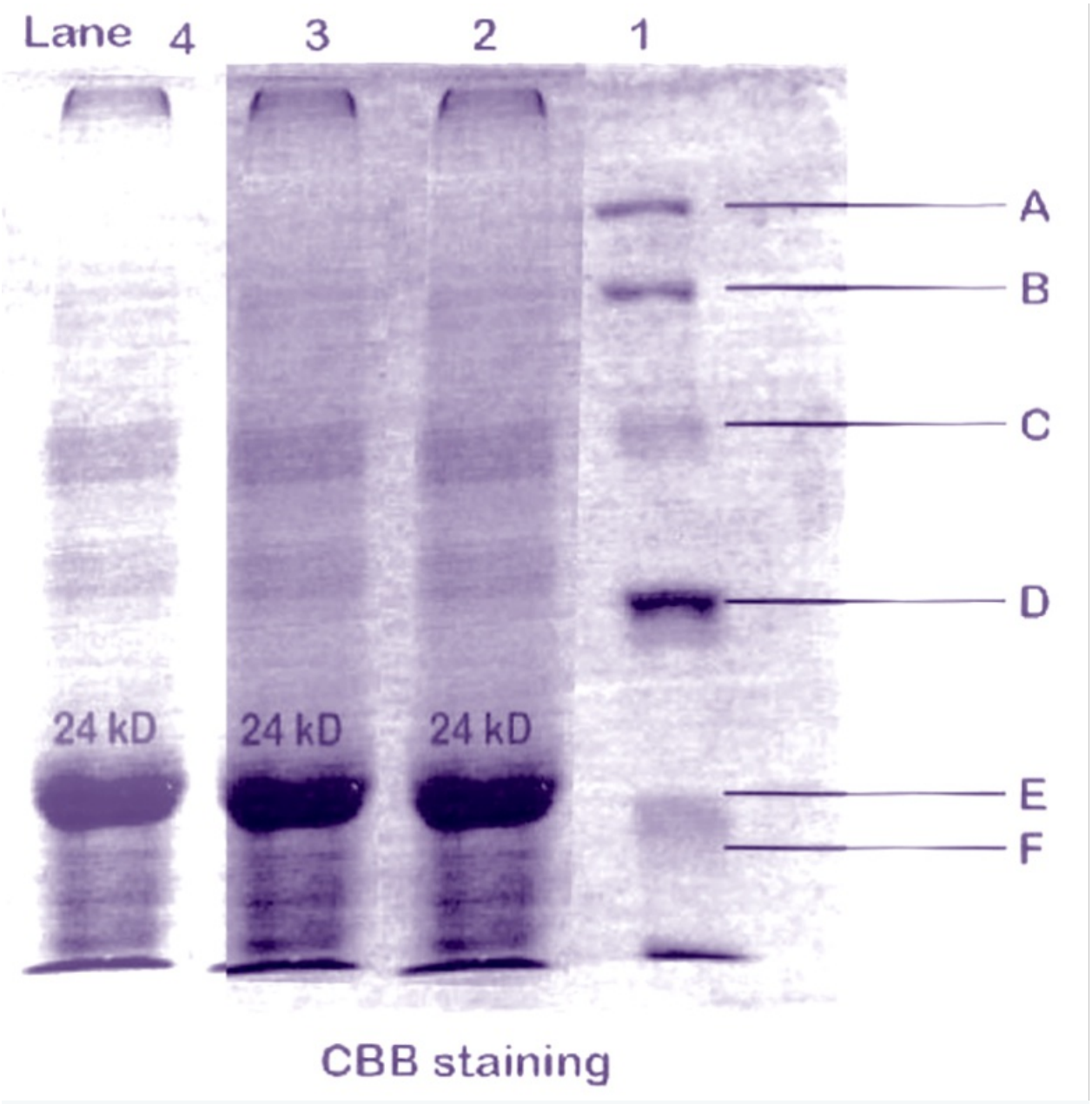

Figure 3. *Analysis of HBsAg release from SDS-PAGE: Lane 1 showing molecular weight of markers; Lane 2 defines HBsAg purity at about 24 kD; HBsAg coated with alginates was revealed in Lane 3; HBsAg loaded nanoparticle is observed in Lane 4*

MTT assay

The cytotoxicity study of prepared formulations revealed dose dependent effect of cell viability. However, LPS-HB-cSLNs was non-toxic to RAW 264.7 cell lines upto highest concentration of 1600 µg/ml in comparison to the control (group). Cell viability was 68.45% at 24 h and 65.45% at 48 h after treatment with LPS-HB-cSLNs (Table 2).

Table 2. Percentage cell viability in MTT assay

Concentration (g/ml)	Control	24 h		48 h	
		Blank cSLNs	LPS-HB-cSLNPs	Blank cSLNs	LPS-HB-cSLNs
0	100.00±0.00	100.00±0.00	100.00±0.00	100.00±0.00	100.00±0.00
12.5	99.11±2.46	99.9±1.69	95.64±1.64	98.9±2.33	94.64±2.22
25	98.64±2.11	98.15±1.99	93.3±1.45	97.15±2.02	92.3±1.56
50	98.73±2.03	95.47±2.01	91.74±1.33	94.47±1.69	90.74±1.33
100	98.25±2.65	89.43±2.70	84.11±2.14	88.43±2.00	83.11±1.07
200	98.17±1.46	85.47±1.65	79.65±1.66	86.47±1.49	78.65±2.66
400	98.15±1.66	84.25±1.33	74.65±2.12	83.25±1.33	72.65±2.16
800	98.1±2.65	80.26±2.16	71.65±2.33	79.26±1.77	70.65±2.64
1600	98.06±2.14	78.65±2.01	68.45±5.46	77.65±1.64	65.45±1.06

Stability studies

Table 3 shows the stability profile of NPs on the storage upto 60 days. The prepared cSLNs was found almost stable during storage. However, the change in particle size of cSLNs at 4±1 and 27±2°C was nominal (207.18 to 287.61 and 305.21 nm respectively) but change in particle size was observed at 4±1 and 27±2°C i.e. 313.82 to 383.09 and 428.53 nm respectively in LPS-HB-AcSLNs on day 60. The average protein content of the LPS-HB-AcSLNs was decreasing (~14%) on storage particularly at 27°C compared to cSLNs (5-6%) through to 60 days, which may be due to leakage of protein from the nanoparticles. By considering the initial protein content of 100%, the percentage decrease of residual protein in NPs was estimated from the formulation upto 60 days of storage. However, this effect was lower at 4°C in comparison to 27°C. In case of storage at 4°C, initial percent residual protein content of blank nanoparticles was found to be 98.31±0.6 and that found to be decreased to 93.46±1.0% after 60 days (Table 4). On the other hand, alginate coated nanoparticles showed 96.35% protein content initially and was found to decrease to 82.13% after 60 days of storage at 27°C.

Table 3. Stability effect of prepared nanoparticles on particle size at different temperatures

Formulations	Particle Size (nm)					
	Initial	10 Days	20 Days	30 Days	45 Days	60 Days
cSLNs	4±1°C					
	207.18 ±3.21	209.14 ±3.07	211.72 ±2.52	234.80 ±2.08	254.05 ±3.32	287.61 ±3.16
	27±2°C					
	207.18 ±3.22	238.43 ±3.04	254.15 ±2.59	276.11 ±2.06	289.5 ±3.38	305.21 ±3.11
LPS-HB-AcSLNs	4±1°C					
	313.82 ±3.75	327.31 ±3.52	348.24 ±3.58	368.18 ±3.01	375.38 ±1.55	383.09 ±3.52
	27±2°C					
	313.82 ±3.71	332.41 ±1.60	344.23 ±3.73	365.37 ±1.62	387.56 ±3.16	428.53 ±3.03

Values were determined as mean ± SD.

Table 4. Stability effect of prepared nanoparticles on protein content at different temperatures

Formulations	Protein content (%)					
	Initial	10 Days	20 Days	30 Days	45 Days	60 Days
cSLNs	4±1°C					
	98.31±0.61	97.89±0.54	97.61±0.34	96.48±0.63	95.02±0.64	93.46±1.01
	27±2°C					
	97.49±0.46	96.25±0.34	95.78±0.74	94.67±0.33	92.13±0.55	91.46±0.81
LPS-HB-AcSLNs	4±1°C					
	97.13±0.55	97.06±0.21	96.26±0.46	95.74±0.51	94.29±0.24	93.38±0.28
	27±2°C					
	96.35±0.62	89.78±0.56	86.49±0.44	85.47±0.57	83.11±0.86	82.13±0.42

Values were determined as mean ± SD.

Immunological response

(Fig. 4) shows cSLNs contents in different body secretions. Anti-HBs titer was highest with LPS-HB- cSLNs in intestinal (3.41 IU/ml), salivary (3.1 IU/ml) and vaginal (2.51 IU/ml) secretions in comparison to control group. (Fig.5) shows anti-HBsAg IgG profile of mice on treatment with different cSLNs formulations upto 56 days. Results showed that LPS-HB-cSLNs exhibited significantly higher anti-HBsAg titer as compared to blank cSLNs. Immunization through oral route produces systemic moreover mucosal immunity. The production of antibodies protected from mucosally transferred HBV. Group I - received PBS; Group II - received blank solid lipid nanoparticles; Group III – received blank HBsAg; Group IV – received HBsAg loaded solid lipid nanoparticle, Group V - received solid lipid nanoparticle loaded with HBsAg and

coated with alginate; Group VI - received LPS-HB-cSLNs; Group VII - received Alum-HBsAg.

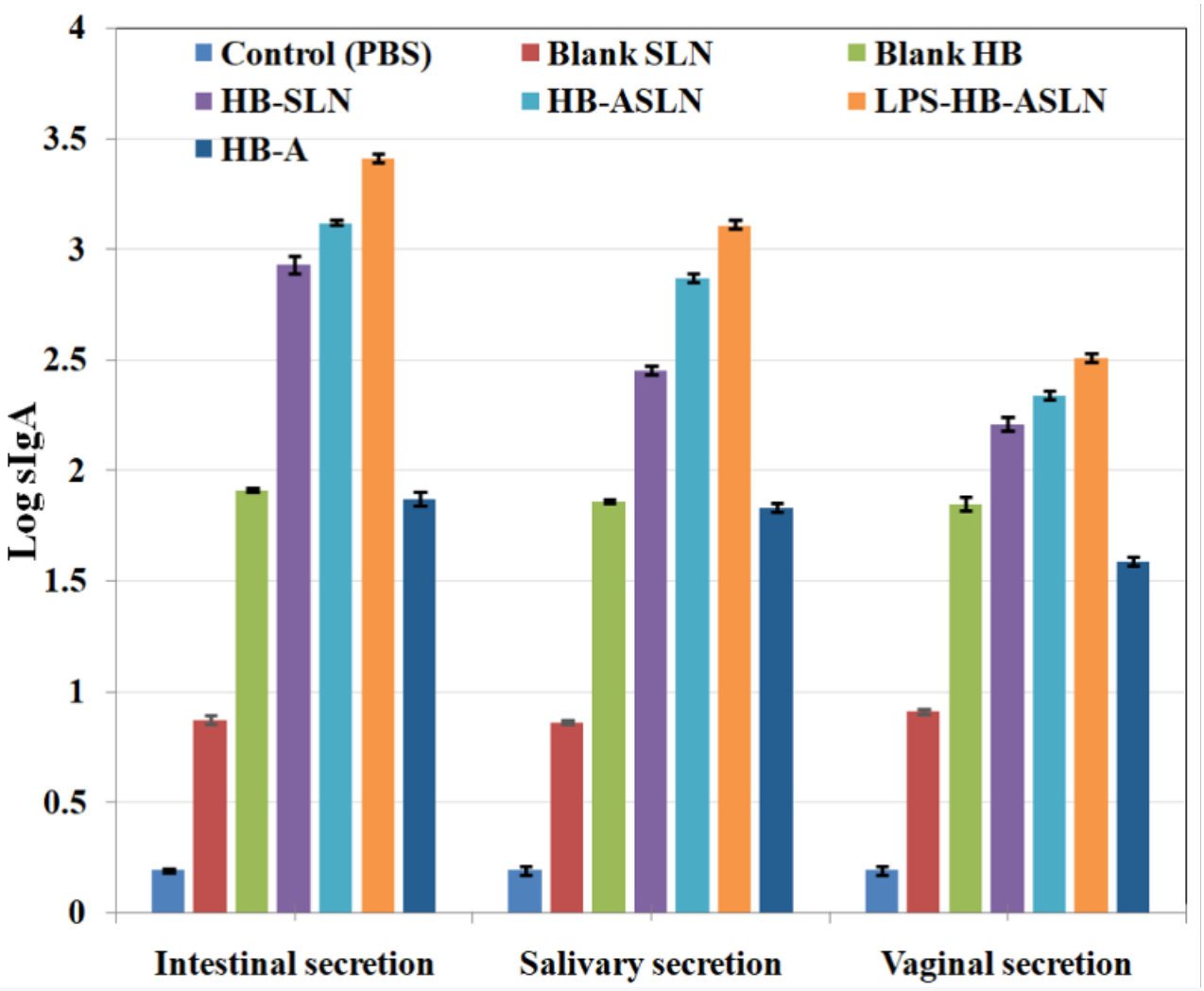

Figure 4. *sIgA profile in mice after administration of cSLNs.Data are represented as mean ± SD (n=6).*

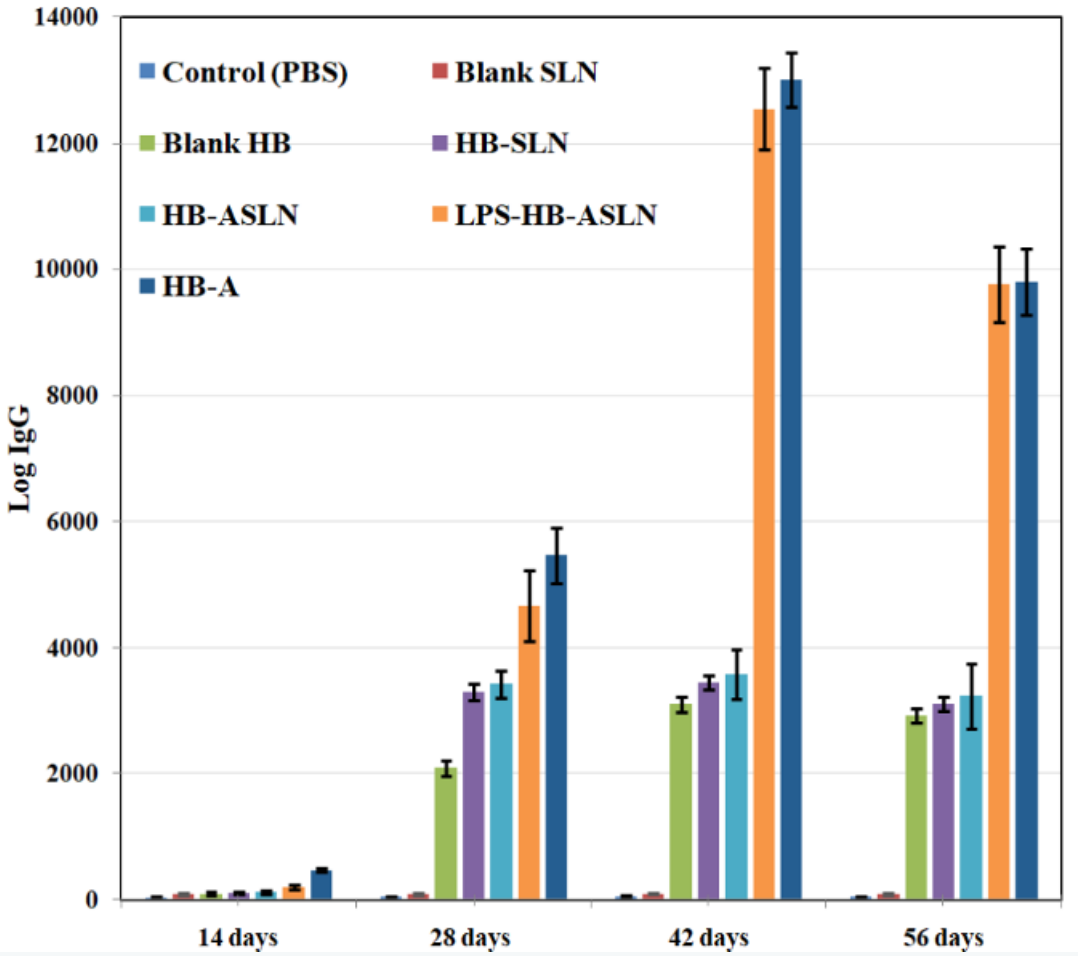

Figure 5. *IgG profile in mice after administration of cSLNs.Data are represented as mean ± SD (n=6).*

Discussion

Vaccination is one of the greatest discovery of medical science to modern era. Researches have been working effortlessly to produce efficient, safe, easily accessible and cost effective vaccine for the human kind. However, vaccination routes for delivery of antigens is a tedious task due to certain disadvantages of all common routes such as inconsistent immune response from intradermal route, pain associated with intramuscular and enzymatic degradation of antigen from mucosal route. Several other problems associated with the existing vaccines are anaphylaxis, possibility of infection at injection site, high cost of vaccines and poor compliance in vaccination programs. On the other hand, immunization at mucosal site may produce long lasting and local immunization against infections. In addition, mucosal vaccines are more convenient and easy to administer with risk of transmitting pathogens. Cationic solid lipid nanoparticles (cSLNs) are the most valuable delivery system for nanoparticles. It has also shown its effectiveness in delivery of non-viral gene[31].

The characterization of prepared nanoparticles revealed that LPS-Alginate coated HB-cSLNs were nanosized and thermodynamically almost stable system. The solvent injection method seems to be effective due to rapid diffusion of solvent between lipid interface and aqueous phase. The surfactant present in the formulation decreased the surface

tension that leads to formation of nano sizes particles[32]. The stability of cSLNs in the internal environment may be due to presence of lactose that gives protection to antigen and efficient delivery of antigen to the desired site[33]. It has reported that colloidal dispersion can be stabilized with high zeta potential (greater than ±30 mV) values due to electrostatic repulsion[34]. Our results were in accordance with the study of*Silva et al.,* (2019) where zeta potential is below +30 mV[35]. Solid lipid nanoparticles were small in size, therefore, providing large surface area making the system effective for delivery of antigens to produce desired immune response[36].

The release pattern of alginate coated SLN revealed that slowly release of protein associated to its surface over an extended period of time was effective due to slow diffusion of entrapped protein[37]. Structural integrity of HBsAg encapsulated in the LPS derived alginate SLN, was determined by SDS–PAGE analysis. The antigen embedded into SLN was found intact and not get damage at the site of M cell contact. The images of SDS-PAGE exhibited that M cell maintained its integrity after binding with SLN and antigen was delivered to desired site of gut after oral administration. Coated particles adhere to longer period with mucosa[38]. The nanoparticle system can improve antigen delivery to the targeted site and also enhance immunogenicity properties that can facilitate the recognition of antigen and its uptake by antigen presenting cells[10]. Cationic solid lipid nanoparticles significantly carrying antigens to induce immunity[39]. In a study it was found that oral vaccination of hydrogel microparticles loaded with antigens was more effective in comparison to intramuscular vaccine in mice model[40]. T and B lymphocytes of the splenocytes have shown the immune functions against Hepatitis B. Similar study was performed by *Sahu et al.,* (2019), targeting colon through solid lipid nanoemulsions encapsulated with enteric minicapsules[2]. Our results were in agreement with the above studies exhibiting effective oral mucosal immunization with cationic solid lipid nanoparticles loaded with HBsAg antigen against hepatitis B. This route has already been proven its effectiveness for delivery of antigens as oral mucosal immunization against hepatitis B[2]. However, this vaccine also showed promising delivery of antigens through pulmonary route to enhance cytokine, mucosal and humoral immunity against Hepatitis B[41].

In our study, LPS-HB-cSLNs exhibited higher anti-HBsAg titer. The principle antibody isotype was sIgA that was produced in all body secretions. Our study was in agreement with the results of *Thomas et al.,* 2009 indicating higher levels of sIgA in body secretions like vaginal, intestinal and salivary and IgG in serum after oral mucosal immunization with HBsAg antigen[42]. Moreover, immune responses produced by LPS-HB-cSLNs was significant among all formulations that were not produced by conventional HB-A. It has reported that anchoring of vaccine with LPS as an adjuvant can effectively trigger immune response against particular pathogen without any causing any toxicity[19]. Our results were in agreement with *Geurtsen et al., 2008* indicating the delivery of vaccine supplemented with whole-cell pertusis antigen anchored with lipopolysaccharide to modify immune responses[43] The vaccine anchored with LPS has already shown its effectiveness in other study[44]. It has reported that in vitro activation of toll-like receptor 3 and 4 can suppress HBV infection[45]. One of the study reported that hepatitis B vaccine adjuvanted with AS04 has shown very promising immune response in dialysis patients[46].

Conclusion

Antigen, HBsAg loaded nanoparticles were prepared for oral mucosal immunization.cSLNs may be an effective carrier system for antigen against Hepatitis B for production of antibodies and induction of both systemic and mucosal immunity, and could be a good alternative to parenteral vaccines for Hepatitis B. Large amount of antigen can be delivered through this delivery system. The association of the HB-ACNPs-LPS to the mucosal M-cells of the mice showed better release in comparison to HBsAg alone. The derivatization of nanoparticles with lipopolysaccharide helped it to target them at the mucosal layer and produce required immune effect against pathogens. The advantage of the developed nanoparticles is the protection of vaccines from gastric acid along with targeting to M-cells. HBsAg present in NPs was also properly stabilized by stabilizers. Thus, HB-ACNPs-LPS anchored with LPS may be an effective vaccine against various infections particularly viral infection.

Ethical Approval

This study was approved by IAEC, Columbia Institute of Pharmacy, Raipur (Regd. No- 1321/PO/ReBi/S/10 CPCSEA)

Author Contributions

Conceptualization: Surendra Saraf, Methodology: Rudra Narayan Sahoo, Writing: Review and Editing: Ashish Kumar Sarangi and Ranjan K Mohapatra, Supervision: Subrata Mallick

Conflict of interest

There is no conflict of interest.

Funding

This research received no specific grants.

Acknowledgements

The authors are acknowledging gratefulness to Prof. Manoj Ranjan Nayak, President, Siksha O Anusandhan (Deemed to be University) for providing other facilities and encouragement.

References

1. [^] *SahuK.K, Pandey R.S. International Immunopharmacology Immunological evaluation of colonic delivered Hepatitis B surface antigen loaded TLR-4 agonist modified solid fat nanoparticles. Int Immunopharmacol. 2016;39:343-352. https://doi.org/10.1016/j.intimp.2016.08.007.*
2. [a, b, c] *SahuK.K, KauravM, PandeyR.S. Chylomicron mimicking solid lipid nanoemulsions encapsulated enteric*

minicapsules targeted to colon for immunization against hepatitis B. Int Immunopharmacol. 2019;66:317-329. https://doi.org/10.1016/j.intimp.2018.11.041.

3. ^*Tan A, Koh S, Bertoletti A .Immune response in hepatitis b virus infection. Cold Spring Harb Perspect Med.2015; 5(8):1-12. https://doi.org/10.1101/cshperspect.a021428*
4. ^*HuiC.K, Lau G.K.Immune system and hepatitis B virus infection. J Clin Virol. 2005;34(1):44-48. https://doi.org/10.1016/s1386-6532(05)80009-5.*
5. a, b*MishraH, MishraD, MishraP.K, NaharM, Dubey V, JainN.K. Evaluation of Solid Lipid Nanoparticles as Carriers for Delivery of Hepatitis B Surface Antigen for Vaccination Using Subcutaneous Route. J Pharm Pharmaceut Sci. 2010;13(4):495-509. https://doi.org/10.18433/j3xk53.*
6. ^*UddinM, AllonA, RoniM, Kouzi S. Overview and Future Potential of Fast Dissolving Buccal Films as Drug Delivery System for Vaccines. J Pharm Pharm Sci. 2019 22:388. https://doi.org/10.18433/jpps30528.*
7. ^*CheccucciE, PiramideF, Pecoraro A, Amparore D, CampiR, FioriC. The vaccine journey for COVID-19: a comprehensive systematic review of current clinical trials in humans. Panminerva 2020; Med https://doi.org/10.23736/S0031-0808.20.03958-0*
8. ^*KourP, RathG, SharmaG, GoyalA.K. Recent advancement in nanocarriers for oral vaccination. Artif Cells, Nanomedicine, Biotechnol 2018;.46:1102-1114.*
9. ^*KaurL, SharmaA, YadavA.K. Recent advances on biodegradable polymeric carrier-based mucosal immunization: an overview. Artif Cells Nanomed Biotechnol. 2018;46:452–464.https://doi.org/10.1080/21691401.2018.1533842*
10. a, b*MohanT, VermaP, RaoD.N. Novel adjuvants & delivery vehicles for vaccines development: A road ahead. Indian J Med Res. 2013;138(5):779–795.PMCID: PMC3928709*
11. ^*Pati, ShevtsovM, SonawaneA. Nanoparticle vaccines against infectious diseases. Frontiers in Immunology. 2018;9:1-16. https://doi.org/10.3389/fimmu.2018.02224.*
12. ^*TanK, LiR, HuangX, LiuQ. Outer Membrane Vesicles: Current Status and Future Direction of These Novel Vaccine Adjuvants. Front Microbiol. 2018;9:783. https://doi.org/10.3389/fmicb.2018.00783*
13. ^*Doktorovova S, Shegokar R, Rakovsky E. Cationic solid lipid nanoparticles (cSLN): structure, stability and DNA binding capacity correlation studies. Int J Pharm. 2011;420(2):341–349. https://doi.org/10.1016/j.ijpharm.2011.08.042.*
14. ^*Kakkar V, Mishra A.K, Chuttani K, Kaur I.P Proof of concept studies to confirm the delivery of curcumin loaded solid lipid nanoparticles (C-SLNs) to brain. Int J Pharm. 2013448(2):354–359. https://doi.org/10.1016/j.ijpharm.2013.03.046*
15. ^*Ganesan P, Ramalingam P, Karthivashan G, Ko Y.T,Choi D.K. Recent developments in solid lipid nanoparticle and surface-modified solid lipid nanoparticle delivery systems for oral delivery of phyto- bioactive compounds in various chronic diseases. 2018;13:1569-1583. https://doi.org/10.2147/IJN.S155593.*
16. ^*Suñé-Pou M, Prieto-Sánchez S, El Yousfi Y. Cholesteryl oleate-loaded cationic solid lipid nanoparticles as carriers for efficient gene-silencing therapy. Int J Nanomedicine. 2018;13:3223–3233https://doi.org/10.2147/IJN.S158884.*
17. ^*Saljoughian N, Zahedifard F, Doroud D, Doustdari F, Vasei M, Papadopoulou B, Rafati S Cationic solid-lipid nanoparticles are as efficient as electroporation in DNA vaccination against visceral leishmaniasis in mice. Parasite Immunol. 2013;35(12):397–408. https://doi.org/10.1111/pim.12042.*
18. ^*Doroud D, Vatanara A, Zahedifard F Cationic solid lipid nanoparticles loaded by cysteine proteinase genes as a novel*

anti-leishmaniasis DNA vaccine delivery system: characterization and in vitro. J Pharm Pharmaceut Sci.2010;13(3):320–35. https://doi.org/10.18433/j3r30t.

19. [a, b]*Battaglia L, Ugazio E Lipid Nano- and Microparticles: An Overview of Patent-Related Research. Journal of Nanomaterials. 2019;1-22.https://doi.org/10.1155/2019/2834941*
20. [^]*Zariri A, Van der Ley P Biosynthetically engineered lipopolysaccharide as vaccine adjuvant.Expert Rev Vaccines. 2015;14(6):861-76. https://doi.org/10.1586/14760584.2015.1026808.*
21. [^]*Borges O, Borchard G, VerhoefJ.C, de SousaA, Junginger H.E Preparation of coated nanoparticles for a new mucosal vaccine delivery system. Int J Pharm. 2005;299(1-2):155–166. https://doi.org/10.1016/j.ijpharm.2005.04.037.*
22. [^]*Jang B, Xu L, Moorthy M.S.Lipopolysaccharide-coated CuS nanoparticles promoted anti-cancer and anti-metastatic effect by immuno-photothermal therapy. Oncotarget. 2017;8(62):105584–105595. https://doi.org/10.18632/oncotarget.22331*
23. [^]*GuptaP.N, MahorS, RawatA, KhatriK, GoyalA, VyasS.P Lectin anchored stabilized biodegradable nanoparticles for oral immunization 1. Development and in vitro evaluation, Int. J. Pharm.2006; 318:163–173. https://doi.org/10.3109/21691401.2013.809726*
24. [^]*Saraf S, Mishra D, Asthana A, Jain R, Singh S, JainN.K. Lipid microparticles for mucosal immunization against Hepatitis B. Vaccine, 2006;24:45-56. https://doi.org/10.1016/j.vaccine.2005.07.053*
25. [^]*GamboaJ.M, LeongK.W. In vitro and in vivo models for the study of oral delivery of nanoparticles. Adv Drug Deliv Rev. 2013;65(6):800–810. https://doi.org/10.1016/j.addr.2013.01.003*
26. [^]*ShihJ.W, GerinJ.L Proteins of hepatitis B surface antigen. J Virol.1977; 21(1):347–357. https://doi.org/10.1128/JVI.21.1.347-357.1977.*
27. [^]*ChoyY.B, ParkJ.H, McCareyB.E, EdelhauserH.F,Prausnitz M.R. Mucoadhesive microdiscs engineered for ophthalmic drug delivery: effect of particle geometry and formulation on preocular residence time. Invest Ophth Vis Sci.2008;49(11):4808-15. https://doi.org/ 10.1167/iovs.08-2515*
28. [^]*KushwahaA.K, VuddandaP.R, KarunanidhiP, SinghS.K, SinghS. Development and evaluation of solid lipid nanoparticles of raloxifene hydrochloride for enhanced bioavailability. Biomed Res Int. 2013;https://doi.org/10.1155/2013/584549*
29. [^]*MehrabiM, DounighiN.M, SorkhabadiS.M, Doroud D, Amani A, Khoobi M, Ajdary S, Pilehvar-Soltanahmadi Y. Development and physicochemical, toxicity and immunogenicity assessments of recombinant hepatitis B surface antigen (rHBsAg) entrapped in chitosan and mannosylated chitosan nanoparticles: as a novel vaccine delivery system and adjuvant, Artificial Cells, Nanomedicine, and Biotechnology. 2018;46:230-240. https://doi.org/10.1080/21691401.2017.1417868.*
30. [^]*YumJ.S, AhnB.C, JoH.J, KimD.Y, KimK.H, KimH.S.Use of pre-S protein-containing hepatitis B virus surface antigens and a powerful adjuvant to develop an immune therapy for chronic hepatitis B virus infection. Clin Vaccine Immunol. 2012;19:120–7. https://doi.org/10.14218/JCTH.2018.00037*
31. [^]*LimeresM.J, Suñé-PouM, Prieto-SánchezS, Moreno-CastroC, NusblatA.D, Hernández-MunainC,CastroG.R, SuñéC, Suñé-NegreJ.M, CuetsasM.L. Development and characterization of an improved formulation of cholesteryl oleate-loaded cationic solid-lipid nanoparticles as an efficient non-viral gene delivery system. Colloids Surf B Biointerfaces,*

2019;184:110533.

32. ^*SchubertM.A,Muller-GoymannC.C Solvent injection as a new approach for manufacturing lipid nanoparticles-evaluation of the method and process parameters. Eur J Pharm Biopharm, 2003;55:125-131. https://doi.org/10.1016/s0939-6411(02)00130-3.*
33. ^*SwathiG, PrasanthiN.L, ManikiranS.S, RamaraoN Solid lipid nanoparticles: colloidal carrier systems for drug delivery. ChemInform.2012 ;43(2):1-16. https://doi.org/10.2147/IJN.S289443*
34. ^*MishraV, BansalK.K, VermaA, YadavN, ThakurS, SudhakarK, RosenholmJ.MSolid lipid nanoparticles: Emerging colloidal nano drug delivery systems. Pharmaceutics, 2018 ;10(4):1–21. https://doi.org/10.3390/pharmaceutics10040191*
35. ^*A.M. Silva, C. Martins-Gomes, J.F. Fangueiro, T. Andreani, E.B. Souto (2019) Comparison of antiproliferative effect of epigallocatechin gallate when loaded into cationic solid lipid nanoparticles against different cell lines. Pharm Dev Technol.2019;11:1-7.https://doi.org/10.1080/10837450.2019.1658774*
36. ^*MukherjeeS, RayS, Thakur RS Solid lipid nanoparticles: A modern formulation approach in drug delivery system. Indian J Pharm Sci.2009;71(4):349–358. https://doi.org/10.4103/0250-474X.57282*
37. ^*MishraN, TiwariS, VaidyaB, AgrawalG.P, VyasS.PLectin anchored PLGA nanoparticles for oral mucosal immunization against Hepatitis B. J Drug Target.2011;19(1): 67–78. https://doi.org/10.3109/10611861003733946*
38. ^*Abd El HadyW.E, MohamedE.A, SolimanO.A, El-SabbaghH.M In vitro-in vivo evaluation of chitosan-PLGA nanoparticles for potentiated gastric retention and anti-ulcer activity of diosmin. Int J Nanomedicine.2019;14:7191–7213. https://doi.org/10.1038/s41598-021-81183-x.*
39. ^*Singh M, Briones M, OttG, O'HaganD Cationic microparticles: A potent delivery system for DNA vaccines. Proc Natl Acad Sci USA.2000;97(2): 811-816. https://doi.org/10.1073/pnas.97.2.811.*
40. ^*ChenX.Y, ButtA.M, Mohd AminM.C.I. Molecular evaluation of oral immunogenicity of hepatitis B antigen delivered by hydrogel microparticles. Mol Pharm. 2019;16(9): 3853-3872. https://doi.org/10.1021/acs.molpharmaceut.9b00483*
41. ^*ThomasC, RawatA, Hope-WeeksL, AhsanF.Aerosolized PLA and PLGA nanoparticles enhance humoral, mucosal and cytokine responses to Hepatitis B vaccine. Mol Pharm. 2011;8(2):405-415. https://doi.org/10.1021/mp100255c*
42. ^*ThomasC, Gupta V, Ahsan F. Influence of surface charge of PLGA particles of recombinant hepatitis B surface antigen in enhancing systemic and mucosal immune responses. Int J Pharm. 2009;379(1): 41-50. https://doi.org/10.1016/j.ijpharm.2009.06.006*
43. ^*GeurtsenJ, Fransen F, VandebrielR.J, GremmerE.R, de la Fonteyne-Blankestijn, BL.J. Kuipers, Tommassen J,Van der LeyP. Supplementation of whole-cell pertussis vaccines with lipopolysaccharide analogs: modification of vaccine-induced immune responses. Vaccine. 2008;26(7): 899-906. https://doi.org/10.4161/hv.22847*
44. ^*ZhangR, RealC.I, LiuC,BabaH.A, GerkenG, LuM, Broering. Hepatic expression of oncogenes Bmi1 and Dkk1 is up-regulated in hepatitis B virus surface antigen-transgenic mice and can be induced by treatment with HBV particles or lipopolysaccharides in vitro. Int JCancer.2017;141(2):354-363. https://doi.org/10.1002/ijc.30742*
45. ^*Wu J, LuM, MengZ.Toll-like receptor-mediated control of HBV replication by nonparenchymalliver cells in mice. Hepatology.2007;46:1769–78. https://doi.org/10.1002/hep.21897.*
46. ^*FabriziF, TarantinoA, Castelnovo C, Martin P, Messa P.Recombinant hepatitis b vaccine adjuvanted with as04 in*

dialysis patients: a prospective cohort study. Kidney Blood Press Res. 2015;40(6):584-92. https://doi.org/10.1159/000368534