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Chylomicron mimicking solid lipid nanoemulsions encapsulated enteric minicapsules targeted to colon for immunization against hepatitis B

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ABSTRACT

The oral route is one of the most convenient routes for drug and/or vaccine delivery. Yet variable nature of gastrointestinal tract due to transient changes in pH, physiology, and flora throughout the gut together with hostile nature of peptide drugs/vaccines when given by this route results in limited success. Colon targeting is a recent area of interest for most of the research among which hard gelatin coated capsules is one such important and useful contrivance. The present study assesses the mucosal immunization with HBsAg loaded lyophilized nanoparticles delivered in the colonic region using enteric coated minicapsules. Designed minicapsules offers better compliance and oral vaccine antigen delivery to the colonic region which involving mucosal exposure thus mimicking the natural pathogen entry in the body. The present study is an extension of our reported work where nanoparticles were administered to the colon through the rectal route. Lyophilized nanoparticles were characterized for particle size, in-vitro release and antigen integrity along with cell uptake study. Particles had ~241 ± 32 nm sizes, flattened yet spherical in morphology. Enteric coated minicapsules were evaluated for size, coating thickness, and dissolution profile. In-vivo immune response assured its immunogenic potential with profound IgG (485 ± 41 mIU/ml) and IgA (885 ± 126 mIU/ml) antibody production as compared to marketed recombinant hepatitis B antigen formulation (Gene Vac-B®) which induce IgG and IgA titer; 1027 ± 62 mIU/ml and 220 ± 11 mIU/ml respectively following well established immunization protocol. Former induced significant mucosal immunity due to the involvement of Common Mucosal Immune System (CMIS). The study supports the workable novel approach for immune protection against hepatitis B.

1. Introduction

Hepatitis B is a severe chronic viral liver disease caused by hepatitis B virus (HBV), transmits similarly to that of HIV but comparatively more infectious [1]. The disease threatens by the annual death rate of 887,000 with 4 million acute infections worldwide. Being as a major cause of liver cirrhosis and hepatocellular carcinoma, the disease infects over 257 million individuals worldwide [2] out of which 20–30% can later develop into liver cancer [3]. Global Burden of Disease study (2010) has ranked HBV infection as a tenth leading cause of death and kept it in a top public health priority [4].

Although its prevalence is declined due to parenteral marketed vaccines, most of the populations in developing countries have limited access to it [5]. The oral route is the most acceptable route for majority population due to its convenience, the possibility of self-administration, avoidance of technical personnel and sterile syringe and pain. The selected region of the gastrointestinal tract (g.i.t.), the colon is a growing area of interest for various vaccine formulations. Mucosal delivery of antigens such as a colonic region of g.i.t., stimulates the common mucosal immune system (CMIS) and thus induce strong antibodies production. Preclinical and clinical trials for colon targeting have been achieved by various routes and formulations in the last few decades; among which one such approach is an enteric coated capsule.

In 1834, gelatin capsules were first patented by Mr. Mothes (Paris) for masking the taste and odor of medicine as an edible container [6]. Although a wide range of advanced polymers for drug targeting is available, the importance of capsules retains as a final proof of drug delivery in intact form to the desired site of g.i.t. if coated with some special polymer. Polymer coated capsules are gaining importance not only for oral vaccination but also for the colonic administration of various new active ingredients [7]. The coating of capsules/drugs using Eudragit as an enteric polymer for colon delivery is widely accepted by which controls site of drug release and based on a combination of its different forms (L-100, S-100, RS, RL etc.).

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Prevalence and death graph of hepatitis B is increasing day by day particularly in developing countries. Although the efficacy of the current marketed vaccine is 95%, the need of oral vaccines are particularly necessary and attractive for two fundamental reasons; first is mucosal exposure (IgA production) and second is for mass immunization. Moreover, this can be realized easily by the success story of oral live-attenuated polio vaccines which almost eradicate its parenteral congeners [8]. In many developing countries, contaminated needles and syringes are serious problems concerning the prevalence of HIV, Hepatitis B and C [9]. So, it's an austere need to develop new generation oral HBV vaccine.

The primary focus of present work was to develop rational and clinically acceptable vaccine dosage form to target colonic mucosa through oral administration which is based on an extension of our previously published work in the same journal where formulation efficacy was assessed following delivery to colon through rectal route [10].

Thus, effective vaccine formulation which may be acceptable for clinical studies was evaluated in the present work to emphasize the significance of targeting colon after oral administration for appropriate systemic and mucosal immune responses.

The hostile environment throughout the g.i. tract (altering pH and presence of proteolytic enzymes) following oral administration of vaccines/peptides degrade/denature the vaccine antigen; targeting vaccine formulation to the colon offers several advantages over other g.i. sites (stomach, small intestine etc.) as larger surface area, longer resident time and lower level of the proteolytic enzyme [11]. Many researchers have already evaluated their formulations and reported this site as an active vaccination site following oral administration [12,13].

Thus the main aim of the present study is to develop stable and clinically acceptable vaccine formulation which after oral administration targeted to colon and presence of discrete colonic lymph follicles taken up antigen and adjuvant together thus stimulate strong systemic as well as mucosal immunity sufficient to produce systemic antibody titer more than seroprotection level.

Further, in our previous study, we determined the ratio of widely available enteric polymers (Eudragit S-100 and L-100 in 4:1) using CCD (Central Composite Design) and developed colon targeted modified nanoparticles (NPs) for oral vaccination of hepatitis B [14]. Preparation of coating solution is a sensitive process and non-aqueous coating solutions are preferred to avoid interaction between water and gelatin which may lead to shell softening and capsule sticking as in case of aqueous dispersion [15]. Therefore, in the present study, minicapsules were coated with a non-aqueous dispersion of the previously optimized ratio of Eudragit polymer for colon delivery.

This study aimed to develop and evaluate lyophilized NPs targeted for colon delivery through Eudragit coated minicapsules which utilized more appropriate and rational route, i.e., an oral route which maintained the intactness of antigenic nanoparticles in minicapsules. NPs composition and preparation were based on our previous study which proved the concept of a colonic region as the prospective site of vaccination [10].

2. Materials and methods

2.1. Materials

Recombinant HBsAg (from genetically modified *Hansenula polymorpha* yeast cells) was gift sample (1.73 mg/ml in PBS pH 7.0) from Serum Institute of India Ltd. (Pune, India). Empty gelatin minicapsules (size 9) and 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC) were provided as gift sample from Torpac®, USA and Lipoid GmbH, Germany, respectively. Monophosphoryl lipid A [MPLA from *Salmonella enterica* serotype Minnesota Re 595] and Glycerol tripalmitate were purchased from Sigma Aldrich, USA and Himedia Laboratories, Mumbai, India respectively. Cholesterol, anti-rat specific IgG, and IgA antibody were obtained from Sigma (Sigma-Aldrich Pvt. Ltd., USA). Bicinchoninic acid (BCA), protein assay kit, was purchased from Genei, Bangalore, India. Fluorescein isothiocyanate (FITC) and Rhodamine were obtained from Sigma–Aldrich, St. Louis, MO, USA. Sodium azide and Foetal calf serum (FCS) were procured from Hyclone, Logan, Utah, USA. Whereas, Fluorescence-activated cell sorting (FACS) buffer and anti CD 11c mAb-FITC were purchased from BD Biosciences, NJ, USA. All other chemicals and reagents were purchased from Merck Chemicals, India of analytical grade whereas deionized distilled water (Milli-Q™ Water system, Millipore Corporation, Massachusetts, USA) was used throughout the study.

2.2. Preparation and lyophilization of NPs

NPs were prepared by solvent evaporation technique as reported by our previous study [10]. Briefly, an equal amount (2.5 g) of tripalmitate (solid core) and DPPC (phospholipid bilayer) and were taken in a round bottom flask with the addition of 100 mg of oleic acid, 100 mg cholesterol and 10 mg tocopherol succinate (antioxidant) to it. The solid fat mixture was dissolved as a clear solution by the addition of 30 ml chloroform to the flask. The resultant solution was subjected to evaporation [Vacuum rotary evaporator (Buchi type), Ultra lab Instruments, New Delhi, India] under reduced pressure to evaporate the organic solvent and to form a thin film. Then 30 ml of phosphate buffer saline (pH 7.4) admixed with 100 µl HBsAg (1.73 mg/ml) and 80 µl MPLA (adjuvant ~ 80 ng) was added to the dry solid fat film to hydrate it and shaken for 30 min until the homogeneous dispersion of all fats were formed in aqueous phase [16]. This process leads to the entrapment of antigen along with adjuvant to the NPs. Prepared NPs were washed three times with the phosphate buffer by centrifugation and subjected for lyophilization.

Preparation and separation of NPs were followed by lyophilization. Aggregation and denaturation of antigen were protected by the addition of mannitol as a cryoprotectant. Briefly, 10% mannitol were added to NPs suspension and mixed well; the mixture was then kept at −20 °C for 7–8 h lyophilization using Freeze dryer (Multitech scientific instrument Ltd., Raipur, India) to obtain the freeze-dried NPs powder. After lyophilization, NPs were packed in an airtight container and subjected to extensive physiochemical characterization.

2.3. Fabrication and filling of minicapsules

Commercially available minicapsules (size 9) were specially designed for rodent use, but it could be fabricated into even more smaller size as much as possible by cutting its body part with a sharp blade in such a way that its cap will completely cover its body part. After this, reduces the size of minicapsules to nearly half of its original size and thus facilitates better oral administration for rodents. Size reduction of minicapsules reduces the filling volume, but the requirement of a very low dose of vaccine fit in the present study. After fabrication to the desired size, minicapsules were filled manually with lyophilized NPs containing a mixture of NPs and mannitol. Uniformity in weight and antigen content were maintained for each minicapsules used for coating.

2.4. Preparation of coating solution and minicapsule coating

The coating solution was prepared by dissolving 240 mg of Eudragit S100 and 60 mg of Eudragit L100 (4:1 w/w) in 5 ml organic solvent mixture containing 1.3 ml of isopropyl alcohol, 1.7 ml of dichloromethane and 2 ml of ethanol. 1% of Polyethylene glycol (PEG 6000) and 2% talc were added to the above mixture as a plasticizer and lubricant respectively. The mixture was thoroughly mixed to form a homogenous thick paste of polymer in which the filled minicapsules were dipped, removed and placed on a Teflon surface. The procedure was repeated for two to three times and finally allowed to dry under a

fan for 15 min. Uniformly and completely dried minicapsules were stored in a cool and dark place. Uniform coating of polymer ratio over minicapsules was determined visually and microscopically. At the end of the coating process, minicapsules were gained about 12 to 14% of total increased weight.

2.5. Determination of physiochemical properties

2.5.1. Physiochemical properties of lyophilized NPs

Morphology and size distribution of prepared NPs were evaluated by electron microscopy as described previously [10]. Briefly, a very thin layer of lyophilized NPs was spread over carbon taps coated aluminum stubs followed by the gold coating (20 nm thickness) which was applied under vacuum over the NPs surface using BIO-RAD POLARAN sputter coater in an argon atmosphere (SEM EVO 50 Carl Zeiss AG, United Kingdom).

Three-dimensional measurement (height and cross section) of NPs was carried out by atomic force microscopy (AFM) at room temperature in a semi-contact mode using NTMDT microscope (Scanning Probe Microscope, Model solver pro-47, Russia) equipped with silicon nitride gold-coated cantilever and tip from NTMDT, sapphire. A very thin layer of NPs was applied over freshly cleaved mica and dried at room temperature. The scanning speed was kept at 1 Hz and images (Fig. 1B and C) were processed with Nova software package from NTMDT.

For release study, 20 mg NPs were suspended in 2 ml of PBS (pH 7.4) to mimic the in-vivo intestinal pH condition of g.i.t. [17] and incubated at 37 ± 1 °C for 5–6 h. At predetermined time intervals, solution aliquots were removed from vortexed Eppendorf tubes followed by centrifugation at 12,000 rpm for 5 min. BCA protein estimation kit was used for determining the antigen content in the supernatant. Triplicate readings were ensured.

2.5.2. Structural integrity and secondary structure conformation

HBsAg was extracted from NPs by dissolving in 2 ml of 5% (w/v) SDS in 0.1 M sodium hydroxide solution. This leads to extraction of antigen and it was loaded in 3.5% stacking gel for processing of electrophoresis (Electrophoresis apparatus, miniprotean 3 cell assemblies Gel Dryer, BIO-RAD, India) at 200 V as per literature [18]. Polyacrylamide gel (15%) was used for separating protein bands whereas Coomassie brilliant blue dye was used for staining protein bands. The gel was removed and the bands obtained were compared concerning their molecular mass [19,20].

Circular dichroism (UVCD) spectra of HBsAg loaded NPs (before and after lyophilization) along with free HBsAg were recorded by JASCO J-815 CD spectropolarimeter (Jasco, Tokyo, Japan) in circular cell with 0.1 cm path-length for data collection. Changes in the secondary structure were determined by recording the spectra in between 90 and 320 nm with correction of solvent background under identical conditions. Triplicate scans for each spectrum were done.

2.5.3. Physiochemical properties of minicapsules

Length of minicapsules was measured before and after fabrication using digital venire caliper and common scale. Three to four minicapsules were measured to obtain a mean value. Coating thickness was determined optically using an optical microscope (Axiocam-Lab1A-ERC-5S, Primostar, Germany). Mean thickness was determined by three to four measurements for each coated and uncoated capsule to be tested.

Coating quality of Eudragit was evaluated by determining the dissolution profile of minicapsules using paddle method at 30 rpm in 1000 ml buffers of varying pH [7] at 37 ± 1 °C. In-vitro release of NPs from minicapsules was performed by suspending six capsules in simulated gastric fluid (pH 1.2) for first 2 h followed by 2 h in simulated intestinal fluid (pH 4.5) and simulated colonic fluid (pH 7.4) for 4–7 h [21]. Samples were withdrawn at prefixed time intervals and release of NPs from minicapsules was analyzed measuring antigen content by BCA method. This is done by solubilizing released NPs in extraction buffer (PBS + 1% TritonX-100) which released loaded antigen from it. Triplicate readings were done for each measurement.

2.6. Fluorescent microscopy

Coated minicapsules containing FITC-NPs were administered orally for fluorescent microscopy examination confirming efficient targeting and uptake of NPs to colonic mucosa and GALT. NPs were prepared by using FITC (0.1% w/v dissolved in DMSO) labeled HBsAg; where HBsAg was mixed gently in it and incubated for 12 h in the dark at 4 °C. [22]. Rats were sacrificed (ether chamber) after 6–7 h of oral dosing; the colon was separated, opened and rinsed thoroughly with PBS (7.4) then with freshly prepared Ringer's solution. Tissue blocks were prepared for microtomy and mounted slides were analyzed under a fluorescent microscope (Axiocam-Lab1A-ERC-5S, Primastar, Germany) after one day.

2.7. Cell uptake studies

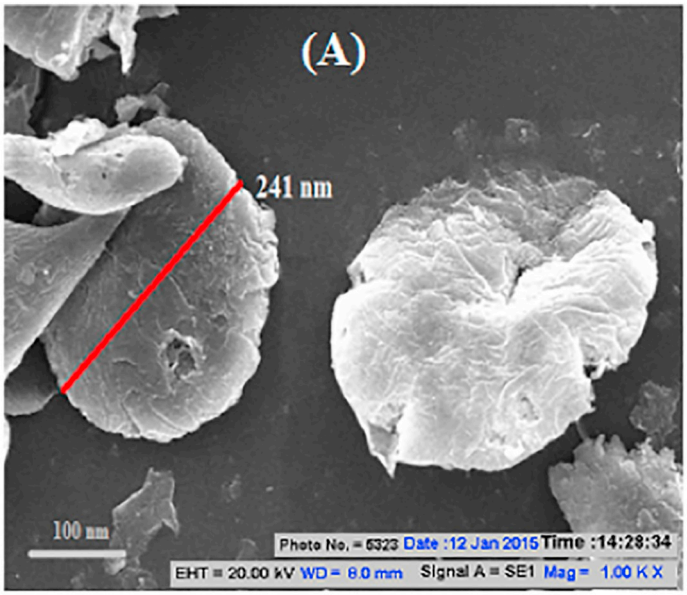
For uptake studies, Bone marrow-derived DCs (BMDCs) were prepared by culturing bone marrow cells in the medium containing 20 ng/ml recombinant granulocyte/macrophage colony stimulating factor (PeproTech, Rocky Hill, NJ, USA), as reported previously elsewhere previously described [23]. Lyophilized NPs were prepared from rhodamine-conjugated HBsAg which were added to the wells of culture plate and mixed well. For conjugation, dye (1 mg/ml in DMSO) was mixed well with HBsAg and kept overnight in the dark at 4 °C which forms an amide bond to amino groups of the protein [24,25]. Uptake was studied at different time intervals of 0, 1, 2, 3, 4, 5, 6 and 24 h. Monocyte-derived dendritic cells (moDCs) were harvested in each time intervals while an excess of formulations was rinsed with ice-cold PBS containing 0.01% sodium azide and 5% FCS, and resuspended in FACS buffer. To further confirm the process of uptake, we also followed a dual-labeling strategy with cells labeled with an anti-CD11c monoclonal antibody (mAb)-conjugated FITC to confirm the gating for DCs with prepared NPs formulation. Flow cytometer measured cell associated phagocytosis (BD Accuri, USA) installed with Cell Quest Software (BD-IS, USA) for measurement of FITC (FL1-H) and Rhodamine (FL2-H). Mock-treated DCs (DCs pulsed with Blank NPs) were used as control [26].

Similarly, CLSM was performed with a Nikon C2 Plus confocal laser scanning microscope to determine the uptake of NPs by moDCs. Dendritic cells (3 × 104/well) were used in 8-well chamber slide (BD Biosciences India Pvt. Ltd., India) at 37 °C for 2 h. Staining of cell nuclei was conducted using 0.5 μg/ml Hoechst stain (Sigma-Aldrich Pvt. Ltd., USA) and cells were incubated with rhodamine-conjugated NPs (10 μg/ml). After 24 h, cells were washed as explained before and then fixed with 4% paraformaldehyde for 30 min for imaging. Blank NPs treated cells were considered as control and used for comparison.

2.8. In-vivo studies

2.8.1. X-ray studies

Institutional animal ethics committee (IAEC) approved the experimental design and research plan after careful assessment of research project regarding animal handling and disposal procedure. Healthy Sprague Dawley rats of either sex (age: 8–10 week, weight: 200–250 g) were used for in vivo study. X-ray imaging of rats was done in every one-hour interval for up to seven hours to visualize and assure the targetability of orally administered minicapsules to the desired site. This study was achieved by barium sulfate half-filled minicapsules coated with a polymer which mimic the actual delivery of NPs to the colon. Rats were kept in a supine position with mild anesthesia while giving minicapsules through the oral gavages.



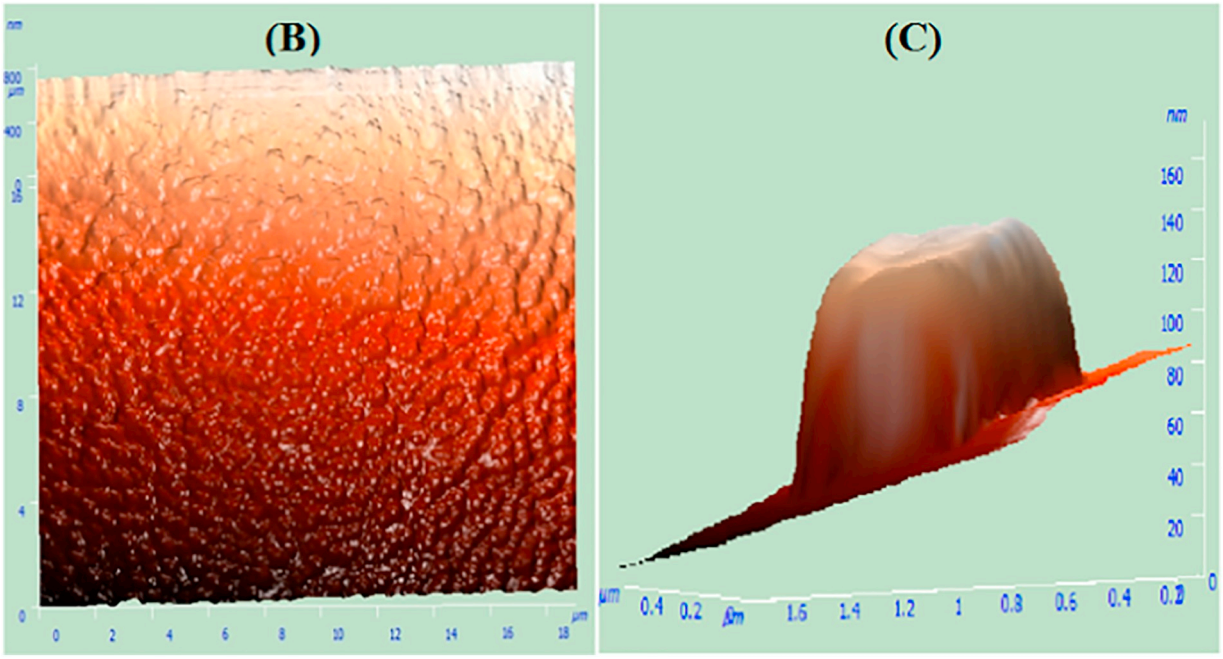


Fig. 1. (A) SEM image of lyophilized NPs showing size, shape and surface morphology (B) semi contact mode AFM image showing 3D structure (C) single NP for better resolution.

2.8.2. Immunological study

Rats were divided into seven groups each containing six (n = 6/group) and immunized as follows: Group I (Control)- Empty minicapsule with mannitol (Oral); Group II- Free HB (lyophilized HBsAg, Oral); Group III- Blank NP (lyophilized blank NPs in minicapsules, Oral); Group IV, V and VI (test groups) receives the optimized and lyophilized NPs in minicapsules given orally with varying doses coded as M-Cap1 (~2.5 μg HBsAg each rat), M-Cap2 (~5 μg HBsAg each rat), M-Cap3 (~10 μg HBsAg each rat) respectively; Group VII (Standard)-Gene Vac-B (10 μg of marketed alum based HBsAg, Intramuscular, Serum Institute of India Ltd., Pune, Maharashtra, India).

Animals were kept for overnight fasting prior to the immunization with free access to water. Various doses were administered via oral gavage of stainless steel to hold the minicapsules attached with 2 ml syringe and filled with PBS for ease administration. This was the first day (0 weeks) protocol and in similar fashion booster dose was also given on 2nd week. Whereas, each animal of all other groups were also treated in the same way with aseptically prepared fresh formulations (lyophilized with 10% mannitol). Single intramuscular immunization followed by the booster dose on 2nd week of the marketed formulation was given to the standard group.

Animals were mildly anesthetized while withdrawing blood samples from retro-orbital sinus on different weeks (0, 1st, 2nd, 3rd and 6th) with the help of glass microcapillaries and collected samples were centrifuged at 5000 rpm cooling centrifuge (REMI, Mumbai, India) for 5 min. Obtained supernatant clear fluid (serum) was separated and

stored at −20 °C. Similarly, colonic washes were also collected and stored for IgA count. For this, animals were sacrificed and their colon was isolated following the longitudinal opening of the colon through the sharp blade. Fecal contents were removed if any and then rinsed in cold PBS. This rinsed solution so obtained was subjected for centrifuge, separated and stored as above and specific IgA content in it was measured by ELISA.

Indirect ELISA with 96 well plates (Sigma-Aldrich Pvt. Ltd., USA) was used for determination of humoral immunity, i.e., serum (IgG) and mucosal (IgA) antibody level. Briefly, antigen coating was applied to each well of ELISA plate with HBsAg (100 µl) diluted with phosphate buffer saline (pH 7.4) and allowed to incubate at 4 °C for overnight. Next day, plates were washed three times with PBS-T (0.0% v/v Tween 20) followed by blocking of remaining free sites of each well by the 100 µl of 2% w/v BSA in PBS-T (Blocking buffer) and again incubated for 2 h at 37 °C. Wells were then filled with 100 µl of serially diluted serum samples, colon washes and control samples after three washings with PBS-T. Plates were then incubated at 37 °C for 1 h and the HRP (Horseradish peroxidase) was added as a secondary antibody whereas TMB (3,3′,5,5′-tetramethylbenzidine) was used as a substrate solution. The reaction was stopped by adding 2 M H_2SO_4 (50 µl) to all wells and the color produced by the reaction was measured at 450 nm.

3. Results

SEM images of lyophilized NPs were recorded for their surface, shape, and size. Images confirmed almost spherical shape and smooth surface of nanoparticles with regular morphology and narrow size distribution (Fig. 1A) showing that lyophilization does not affect its structural morphology but slightly increased in particle size was observed (~245 ± 32 nm) as compared to a previous report [10]. However, lyophilization did not affect antigen loading capacity and discreetness of NPs.

Semi contact mode AFM images of dried NPs are presented in Fig. 1B and C as its slightly flattened but smooth and circular shape with average height and diameter of 160 nm and 250 nm respectively. Dimensions of discrete NPs obtained from AFM studies correlates to its scanning microscopic images.

HBsAg release study from NPs was determined at various time intervals in PBS (pH 7.4) 2–11% HBsAg released in first 2–3 h and reached a maximum between 5 and 6 h (17.5% ± 1.45%) then release was decreased (Fig. 2A). Release pattern was slightly different from non-lyophilized NPs where flat and steady release curve was obtained as reported in our previous paper [10].

SDS PAGE gel images clearly showed distinct bands of proteins in gel matrix separated with respect of their size and weight. Extracted HBsAg (Lane 3) showed slightly thin band in comparison with a molecular marker (Lane 1) and free HBsAg (Lane 2) because of its low concentration in NPs but enough to show its structural identity and integrity (Mw ~ 24 kDa) (Fig. 3). SDS PAGE gel image also relates the preservation of antigen linear conformation during formulation steps.

CD spectra of free HBsAg (Fig. 4) showed two strong negative bands at 216 and 222 nm, whereas non-significant difference was seen with extracted HBsAg. As reported by the Greiner et al. [27], these are the two characteristic bands for HBsAg which confirms antigenic structure was preserved during formulation.

Fluorescent microscopy of colon tissue (Fig. 5) clearly exhibits and confirms the efficient targeting and uptake of FITC conjugated NPs. Although the histological image of treated group showed limited fluorescence because of a low dose of antigen though uptake was confirmed as no such fluorescence in the control group was observed. Further, colon delivery of NPs was assured and directed by minicapsules as histology of another part of g.i.t. doesn't show fluorescence. Whereas, colon patch epithelia in rat colon contain electron-dense cells richer with dendritic cells together with a bunch of B cells and T cells construct optimal mucosal immune response [10]. In contrasts, an

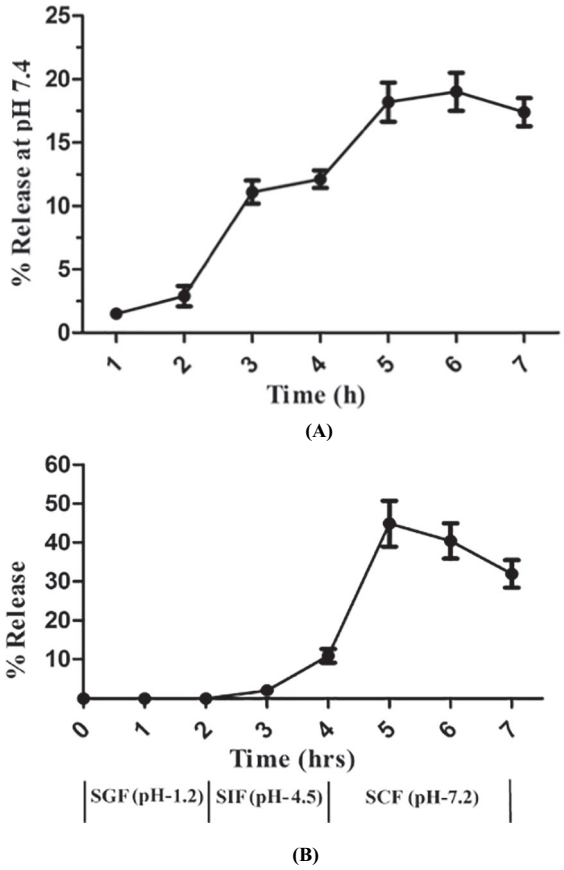


Fig. 2. Release studies (A) showing antigen release from lyophilized NPs (B) in vitro dissolution profiles of coated minicapsules in different simulated g.i.t. fluids over a period of time (mean ± SD, n = 3). [SGF: simulated gastric fluid; SIF: simulated intestinal fluid; SCF: simulated colonic fluid].

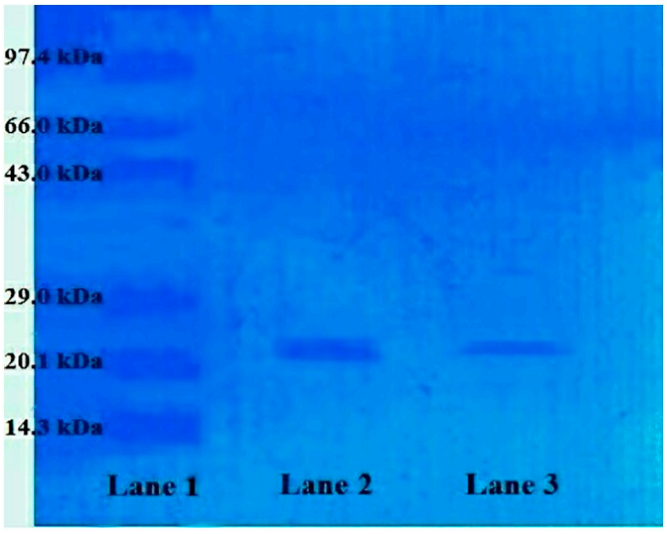


Fig. 3. SDS-PAGE gel photograph (Lane 1: molecular marker; Lane 2: free HBsAg; Lane 3: extracted HBsAg). Molecular marker composed of Phosphorylase b: 97.4 kDa; bovine serum albumin: 66.0 kDa; ovalbumin: 43.0 kDa; carbonic anhydrase: 29.0 kDa; soya bean trypsin inhibitor: 20.1 kDa.

abundance of M-cells and GALT (Gut associated lymphoid tissue) in colonic region facilitates transcytosis of NPs for efficient induction of immune response in human beings [28,29].

The confocal laser scanning microscopy (CLSM) was used to assess

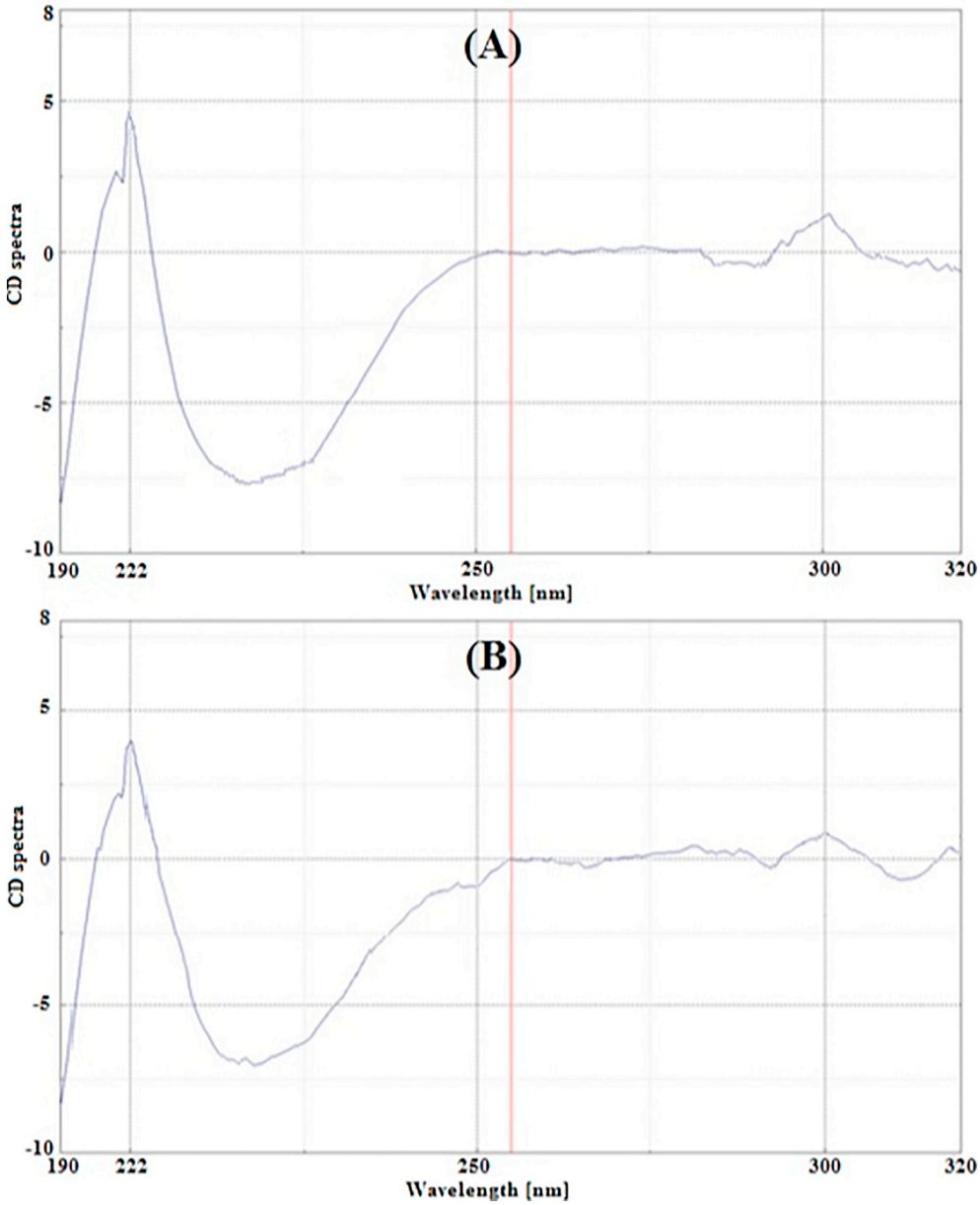


Fig. 4. UV CD fluorescence spectra of (A) pure HBsAg and (B) lyophilized NPs. It shows non-significant changes in the CD spectra, as compared to pure HBsAg particles.

targeting potential of lyophilized NPs. Enhanced uptake was clearly shown in Fig. 6 by rhodamine-conjugated HBsAg-NPs (~5–6 fold higher uptake) as compared to blank NPs where the negligible binding was seen. Uptake was significant for HBsAg-NP at 4 h (52.61% ± 2.50%) as compared to DCs pulsed with soluble HBsAg (4.29 ± 0.51) as revealed by using in vitro flow cytometry. Thus, dye-conjugated HBsAg NPs after 24 h incubation showed increased uptake rather than adherence to the plasma membrane. Moreover, it indirectly ensures the effective formulation of NPs so that better cell uptake. The further conformation of above findings done via anti-CD11c mAb–conjugated FITC for DCs and rhodamine-labeled HBsAg-NPs using dual-labeling strategy also showed similar results (Fig. 7).

3.1. Physiochemical properties of minicapsule

Length of procured minicapsules was 9.05 ± 0.02 mm while that of fabricated were 5.46 ± 0.08 mm (uncoated) and 5.58 ± 1.13 mm (coated) as measured by digital venire caliper (Fig. 8). Thus it is clear that the size was reduced to almost half in respect of length while diameter remains the same. Eudragit coating of minicapsules increases nearly 30–40% of its actual thickness while a repeated coating of up to five times contributes about 50% thickness (Fig. 9D). Although complete coating was achieved by such polymer combination but overall texture was somewhat uneven at micron level as shown in cross-section image of minicapsules (Fig. 9). Whereas, coated minicapsules were free from any pores or cracks ensuring its enteric integrity.

Dissolution of minicapsules was estimated in respect of antigen release from NPs. NPs released slowly from its gelatin shell. Enteric coating of minicapsules was proved by dissolution studies where no release of NPs was noticed at pH between 1.2 and 4.5 (SGF and SIF) whereas 40–45% ± 4.2% releases at pH 7–7.4 (SCF) (Fig. 2B). Movement of minicapsules from upper (acidic) to lower (alkaline) part of g.i.t. leads to a pH shift which ionizes carboxyl group of Eudragit coating and thus weakening of its coating ability [30]. This was followed by swelling and rapture of capsule shell and ultimately diffusion of NPs from it. Collected NPs showed 16.2% ± 1.8% antigen release from it.

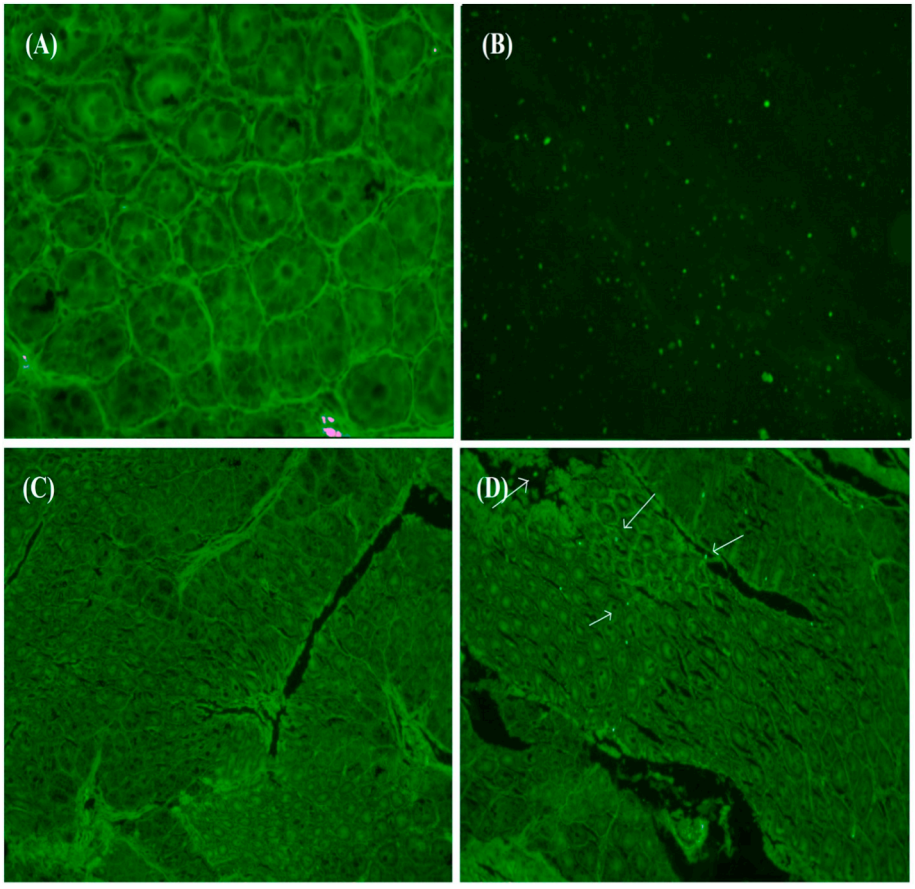


Fig. 5. Fluorescent microscopy of (A) untreated colon (B) FITC entrapped NPs (C) control group received PBS (D) colon tissue showing NPs uptake administered orally (treated group).

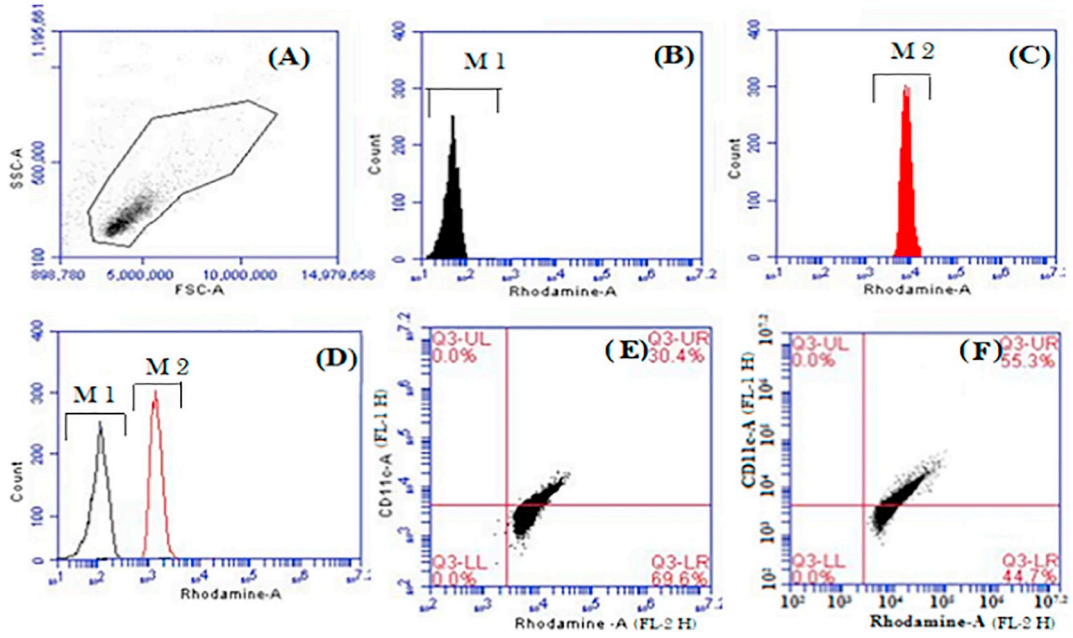


Fig. 6. (A) and (D) spectral bioimaging of moDCs with Hoechst stain showing fluorescence. (B) and (E) are cells after 4 h incubation with Blank and HBsAg NPs respectively conjugated with Rhodamine. (C) and (F) are overlapped images. Fluorescence clearly showed the better uptake of HBsAg NPs as compared to blank.

3.2. In-vivo studies

X-ray studies from 0 to 7 h (1 h time interval) confirmed the movement and targeting of minicapsules to the desired site. Fig. 10 clearly showed the intactness of minicapsules up to 3 to 4 h, i.e., an acidic pH while the slight release was observed after 4 h when it completely reaches in alkaline pH of the colon. Thus, the result outcomes of this study proved the minicapsules for assured delivery of NPs

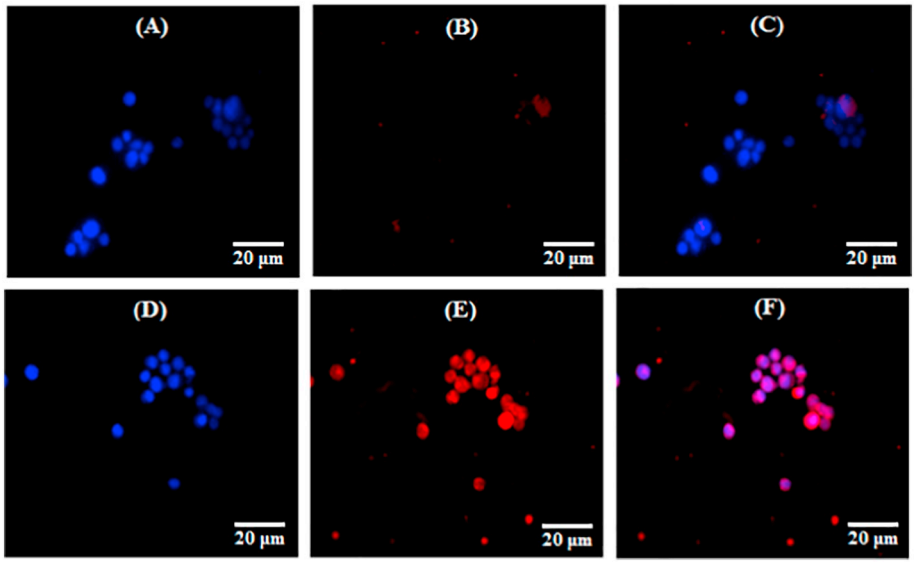


Fig. 7. Measurement of uptake of HBsAg NPs conjugated with Rhodamine and DCs labeled with anti-CD11c mAbs conjugated with FITC measured by flow cytometry. Cell-associated fluorescence dual-labeled (i.e., positive for both FITC [FL1-H] and Rhodamine [FL2-H]) was considered. Triplicate readings were taken for represented plots and values are expressed as mean ± SE for M2 zone (uptake). (A) FSC/SSC dot plot showing a cluster of DC population. (B) DCs pulsed with soluble HBsAg after 4 h of incubation (4.29 ± 0.51). (C) Uptake of HBsAg NPs by human DCs following 4 hour incubation (52.61% ± 2.50%). (D) Increased uptake was observed when compared between DCs pulsed with soluble HBsAg and HBsAg NPs after 4 h of incubation. (E) Uptake of HBsAg NPs by human DCs labeled with anti-CD11c-FITC with following 1 hour incubation. (F) Uptake of HBsAg NPs by human DCs labeled with anti-CD11c-FITC following 4 hour incubation.

to the colonic region in between 4 and 5 h. X-ray study was followed by in-vivo immune response studies where the effectiveness of prepared NPs was ascertained. Our previous research on colonic site has already added on the hypothesis that colon has strong potential as an immunogenic site in g.i.t.

Collected serums of treated animals were used for anti-HBs serum IgG antibody titer (log 10 mIU/ml) where the immune responses were compared among the test groups. Marketed Gene Vac-B produces slightly higher immune response in case of IgG as compared with all test groups (M-Cap1, M-Cap2 and M-Cap3) as observed in various immunization weeks (Fig. 11). Among all test groups M-Cap3 was better in response, producing 485 ± 41 mIU/ml; *$p < 0.05$ as compared to Gene Vac-B (1027 ± 62 mIU/ml). Whereas, IgA response produced by M-Cap3 was considerably higher (885 ± 126 mIU/ml; **$p < 0.01$) as compared to the marketed vaccine in which mucosal exposure was lacking thus negligible response was seen (220 ± 11 mIU/ml; Fig. 11B). Moreover, booster doses in various groups produced a progressive immune response as compared to priming doses (Fig. 11C and D). This result signifies the importance of colonic administration of minicapsules which utilize particularly colon

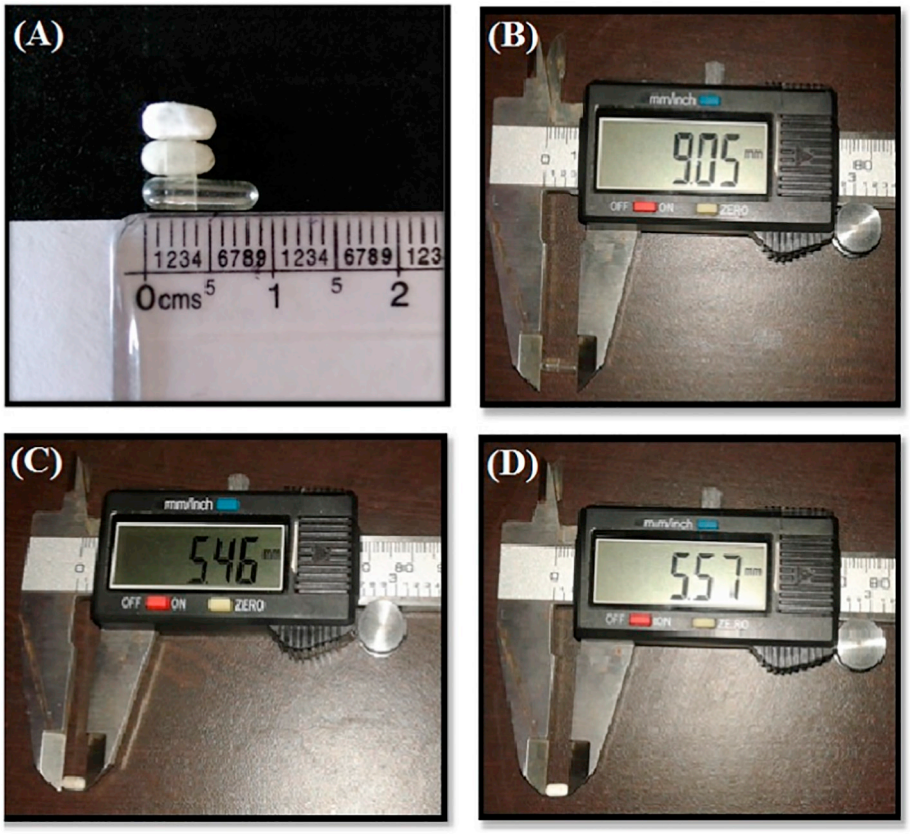


Fig. 8. Dimensions of minicapsules: (A) simple scale measurement (B) digital venire caliper measurement of non-fabricated minicapsule (C) uncoated and fabricated minicapsules showing reduced size to almost half (D) fabricated with Eudragit coated minicapsules.

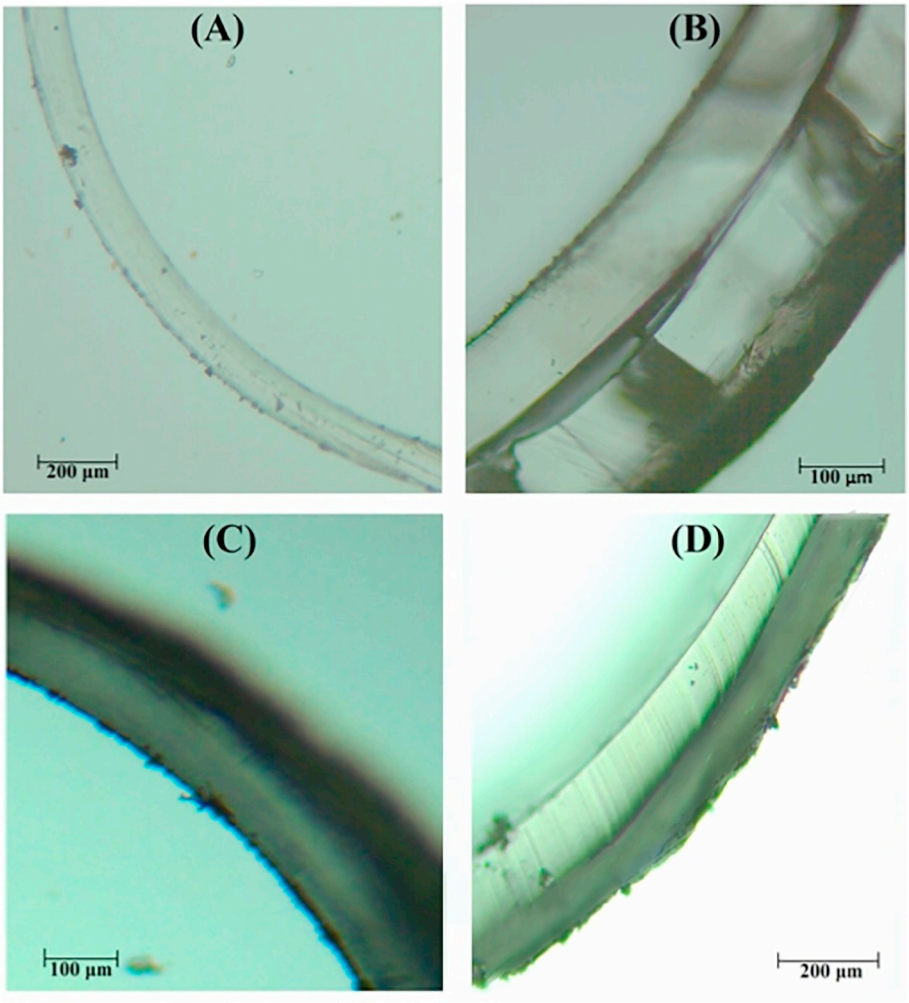


Fig. 9. Transverse section of minicapsules: (A) uncoated (B) joint area with coating (C) single coating (D) multiple coating of up to five times.

mucosa and GALT thus produces approximately two to three-fold more mucosal immune response as compared to the marketed vaccine. Mucosal IgA has already been reported to play a significant role in pathogenic protection as well as mucosal homeostasis both in animals and human beings [31,32]. Present formulation is not lacking from cellular immune response (IL-2 and IFN-γ) which was also quite acceptable as shown in our previous paper [10].

4. Discussion

Although an oral vaccine for hepatitis B is not a new initiative; several researchers have already formulated oral vaccines for it [33]. The present study was focussed on the use of enteric-coated minicapsules for delivery of NPs directly to colon region in an intact form by oral administration. Minicapsules can be easily targeted to colon through the enteric coating and can be a suitable carrier system for delivery of any drug in intact form. Moreover, targeting to colon leads to profound and long-lasting immune response through mucosal antibody production (IgA) as reported previously [10,14]. Based on these studies, the practical implication of that concept using capsule as oral delivery of formulation is realistic and implied in clinical studies as well. Being richer with lymphoid tissue and M-cells, colon region still underutilized vaccine target site because of its targeting and penetrating challenges.

Obtained SEM images of lyophilized NPs showed its morphological similarity with that of freshly prepared NPs. The result confirms that the spherical shape and smooth surface of NPs remain unchanged with a slightly increased average size range of ~240 nm which may be due to the presence of cryoprotectant (Mannitol). Moreover, its 3D morphology was confirmed by AFM studies where it was found to be very similar to SEM dimensions with nano-size range. Thus, the overall difference in size and shape was negligible with that of our previous report with similar NPs and morphology was conserved even after lyophilization.

Release pattern of lyophilized NPs was slightly changed by lyophilization. Burst release of antigen after 2 h was noticed which may be due to the formation of pores and minute fracture over NPs surface. Increased solvent attack and quick diffusion were noticed as compared to freshly prepared NPs. Absence of any special polymer over NPs also hold up its easy and quick release as supported by others [34].

Clear and distinct bands at same loci were obtained confirming structural integrity of antigen. Being an easy and rapid method for protein analysis it also ensures that the formulation step did not cause any significant antigen aggregation and degradation [35]. However, the result obtained also supports the biological intactness of antigen related to its immune response probability. Application of UVCD for qualitative secondary structure conformation study of HBsAg confirms that no such significant changes were observed at 216 and 222 nm bands. It also supports the intactness of active sites of antigen.

Evidence of BMDCs uptake of HBsAg NPs in hourly incubation subjected to flow cytometry confirms and supports the antigen-specific immune responses as significant uptake was shown compared to soluble HBsAg.

Simultaneous visualization of minicapsule at different time intervals

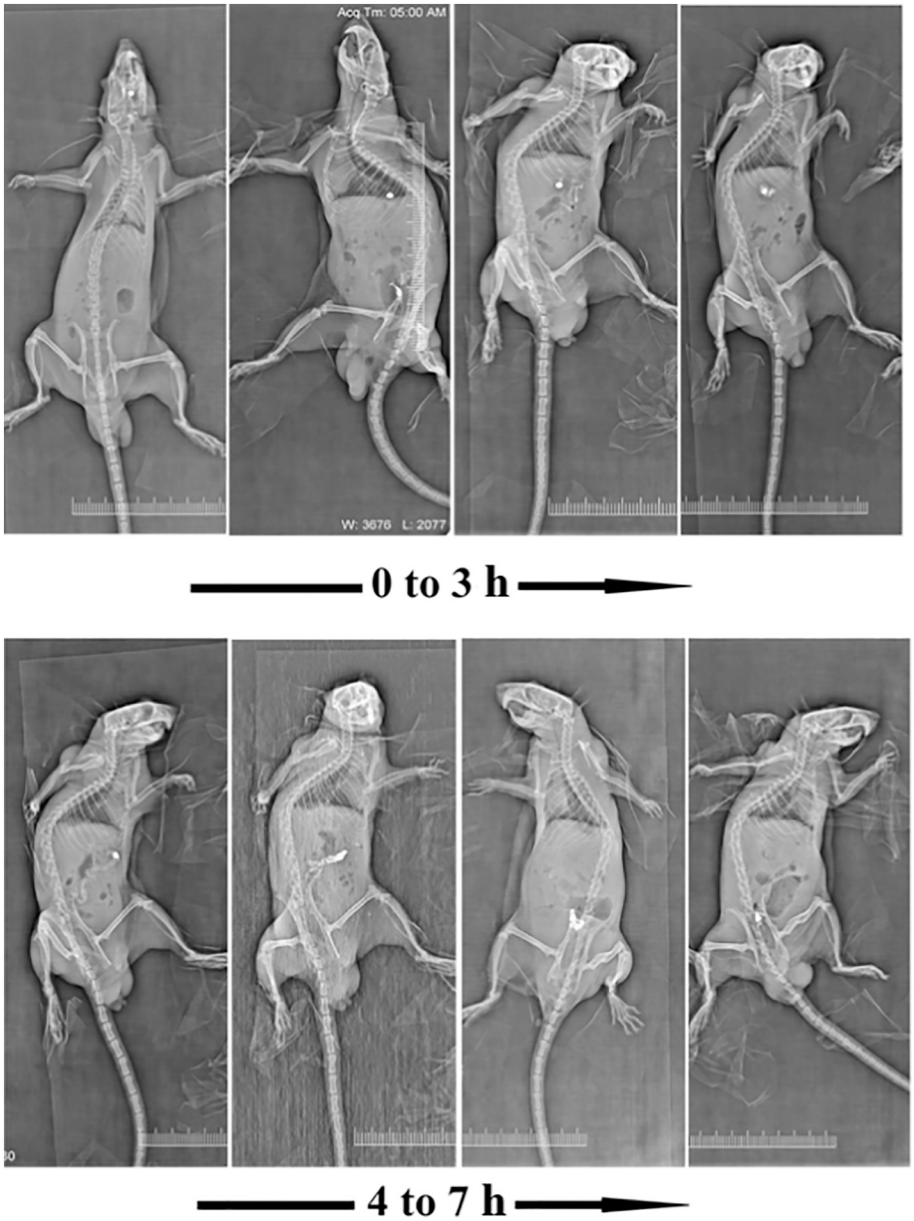


Fig. 10. X-ray images of the rat at different time intervals confirming targeting of orally administered coated minicapsules filled with barium sulfate.

through the GI tract was determined using X-ray contrast agents. As shown, the X-ray result was quite acceptable and confirmed that Eudragit coated minicapsules was able to deliver its content in the alkaline pH of the colon [36]. Fig. 11 represents that IgA immune response was significantly increased in the group received optimized prepared formulation by oral route whereas serum IgG titer was highest in the group received marketed formulation by intramuscular route. Moreover, maximum anti-HB antibodies production was observed at 3rd week. Lacking IgA response in marketed one is due to inefficient mucosal exposure where immune cells are abundant. Booster dose induces the generation of prolonged immune response where activation of memory B lymphocytes is invo6lved. Initially, exposed B lymphocytes are sensitive to the further attack of particular antigen and being as precognitive as well as sensitive it capably proliferates, differentiate and produce specific antibody [37,38].

M-cells or membranous cells or microfold cells or modified epithelial cells help in processing and transport of antigen to Peyer's patches causing profound immune stimulation and antigen-specific B cells release [39], similar in action but morphologically different antigen capturing epithelial cells were also found in rat discrete colonic lymphoid patches [40]. Additionally, fluorescent microscopy used for visualization of NPs uptake in the colonic mucosal cell was the strongest facet of the successful formulation. Furthermore, MPLA (adjuvant) and DPPC make it structurally similar with natural pathogen as discussed and proved in our previous work [10,41].

Oral vaccines generate IgA as the first line of defense in gut together with seroprotective level of IgG and other immune effector molecules and cells [interferons, interleukins, activated B and T cells etc.]. This assures the generation and contribution towards adaptive immunity of gut [42]. Several researchers have already delivered their different vaccine formulations to the colonic and other mucosal targets; explored its immunogenic potential and found significant stimulation of both type of immunity (mucosal and systemic) [43–46]. MPLA is first approved adjuvant from TLR agonists category and used for mass vaccination for human [47]. MPLA is most safe and widely used adjuvant, compatible with variety of antigen [48,49].

Being the significant antibody of mucosal secretions and the first line of defense for pathogens entering from the mucous membrane, sIgA

plays an important role in the clearance of antigenic material from intestinal lumen [50]. Research showed that about 15% of the total immunoglobulin content of the body is sIgA as secreted nearly three to five grams from git, every day [51,52]. Thus, gut plays an important role in generation and proliferation of Common mucosal immune system (CIMS) comprises of Gut-associated lymphoid tissue (GALT) which not only protects the body from gut invasion but also augments systemic immunity (both Th1 and Th2 immunity) [53,54]. However, strong systemic immunity is generated following oral vaccination when appropriate doses of antigen and adjuvant are targeted to localized mucosal lymph follicles (Peyer's patches in the intestine and colonic patches in the colon) that further reduce chances of immune tolerance [55].

Gelinas et al. demonstrated a different correlation between humoral responses of HBV vaccine with its cellular arm of immunity including significant IFN-γ and IL-10 secretion. This study also signifies the immune protection of low or non-responders by some alternative or supplementary mechanism correlated with vaccination [56]. In the present study, we also observed the simultaneous stimulation of both humoral and cellular immunity from which we can conclude that they are correlated.

These findings protract the development of colon targeted oral vaccine which can be promising especially for developing countries where mass vaccination and patient compliance is most important.

Human git responds to a wide range of antigen for complex mucosal immunity including both IgG and IgA, along with T cell activation. Mucosal IgG contributes a significant role in the prevention of pathogenic diseases even HIV transmission if administered passively [57]. Moreover, our previous study accomplished with the activation of T-cells which assure Th1 immunity i.e. IFN and IL-2. Since our present study is just exploration of clinically acceptable vaccine dosage form design based on our previous work, it also showed consistency with T cell activation.

Thus, all these studies supports that coated minicapsules might be an interesting delivery vehicle for new generation antigen and adjuvant to the desired site of g.i.t. It would be interesting to bring such results at the molecular level and transforming it for large scale and/or clinical validation as it provides a reason for cost-effective vaccine alternative

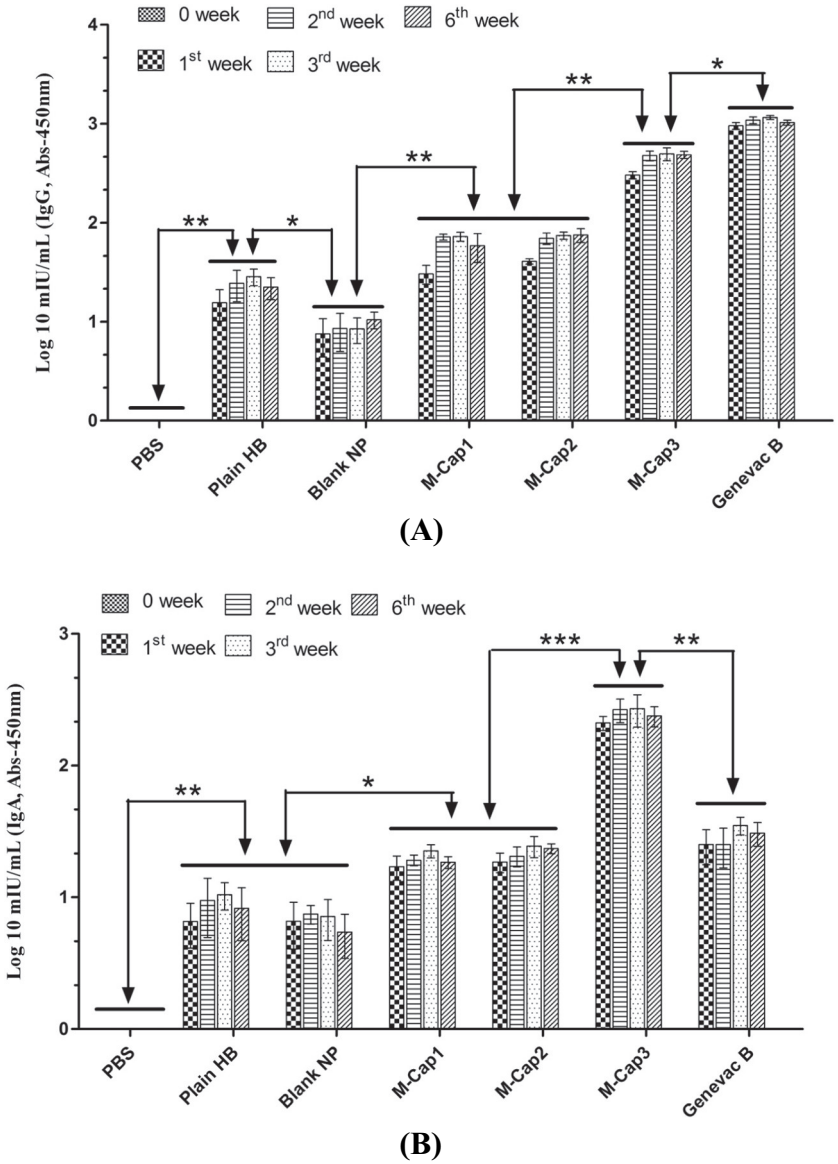
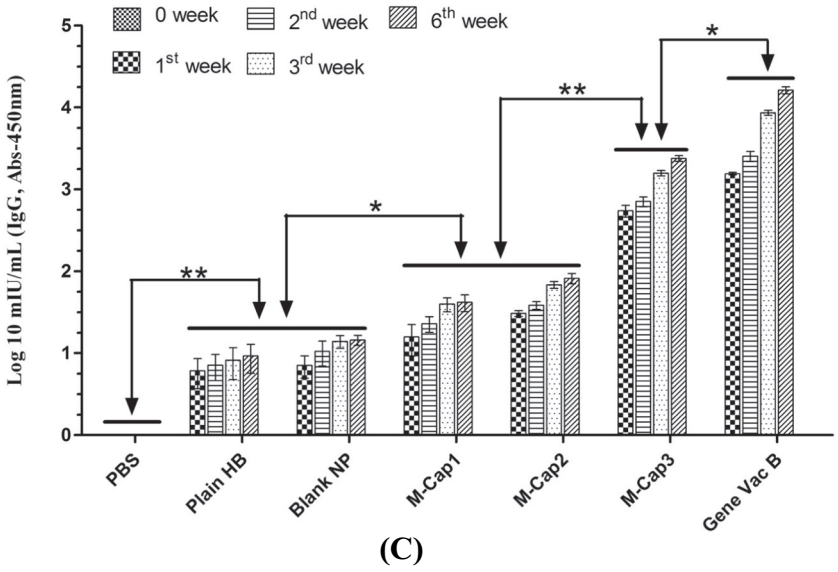
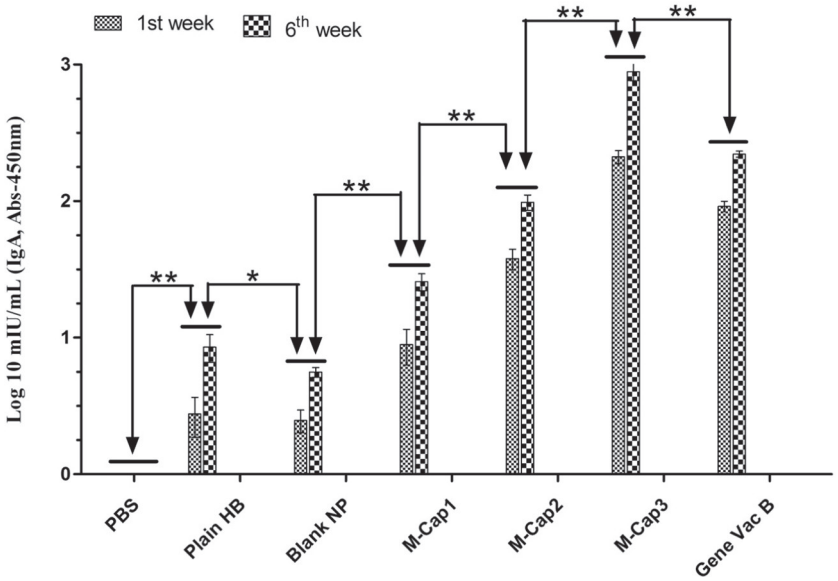


Fig. 11. (A) Serum immune response (IgG) and (B) Mucosal immune response (IgA) as determined by indirect ELISA (C) and (D) are booster dose responses of IgG and IgA respectively [values are expressed as mean ± SEM (n = 6), ***p < 0.001 was considered as significant whereas **p < 0.01, *p < 0.05 and ns N > 0.05].



(C)



(D)

Fig. 11. (*continued*)

to the conventional vaccines.

5. Conclusion

Results obtained from the present study showed that lyophilization was not detrimental to immune active NPs characteristics regarding intactness in structural integrity and immunogenicity of antigen, and found beneficial to formulate in minicapsules. Minicapsules have much to offer as a potential delivery system by providing the possibility of personalized medicine as well as deciphering immunology, ultimately may build better and effective therapeutics. It further requires to be tested in higher animals and clinical affirmation. The storage stability studies also need to be done before this formulation accepted for further studies.

Declarations of interest

None.

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