INTERNATIONAL JOURNAL OF PHARMACY AND ANALYTICAL RESEARCH

ISSN: 2320-2831

IJPAR |Vol.8 | Issue 4 | Oct - Dec -2019
Journal Home page: www.ijpar.com

Research Study *Open Access*

Development, Characterization and in-vitro Evaluation of HBsAg loaded PLGA microspheres stabilized with protein stabilizers

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ABSTRACT

The objective of this study is to develop a stable single-dose vaccine based on recombinant hepatitis B surface antigen (HBsAg) in PLGA microspheres in which HBsAg is stabilized using protein stabilizers and an antacid and to demonstrate its potential as mucosal adjuvant for vaccines. Preliminary studies like Surface morphology, size distribution analysis, percentage entrapment efficiency and *in-vitro* analysis were performed. In the entire study tetanus toxoid (TT) was used as model antigen and HBsAg was used as candidate antigen. Stability studies were carried out for the microspheres with and without stabilizers at 4^0 C and 37^0 C with RH 60 ± 5%. Percentage entrapment efficiency and EIA / Protein ratio were investigated. The size distribution analysis suggested that the particle size of individual microsphere was remained same in the range of 1-10 µm. The percentage entrapment efficiency of the model antigen loaded microspheres and the candidate antigen loaded microspheres were up to 80 on 16^{th} day and was dropped to 65 on 19^{th} day. The integrity of the antigen was assessed by western blot analysis and was found to be unaltered up to 16 days at 37^0C. The *in vitro* immunogenicity of encapsulated HBsAg was evaluated by EIA assay and the EIA/protein ratio of both the antigen was up to 0.9±0.2 on 16^{th} day and was dropped to 0.7±0.2 on 19^{th} day. To conclude the HBsAg containing PLGA microspheres were found to be stable for 18 months, 16 days and 84 days respectively at 4^0 C, 37^0 C and 25^0 C.

Keywords: Hepatitis B Vaccine, PLGA microspheres, r HBsAg, Enzyme Immuno- Assay and Western Blot.

INTRODUCTION

Hepatitis B virus (HBV) remains an important health problem worldwide and often referred to as silent killer. Infection with HBV causes acute as well as chronic necro inflammatory liver disease and many HBV eventually develop into serious complications such as liver cirrhosis or hepatocellular carcinoma. There are about 300 million chronic HBV carriers worldwide and these carriers represent a permanent source of HBV infection. It is well-established that the humoral immune response to HBV envelope antigen leads to protection against infection. In contrast, cellular immune response has been shown to be one of the most important factors contributing to virus elimination from infected hepatocytes and play an important role in the subsequent development of chronic liver disease [1].

The hallmark of vaccine delivery is to produce a vaccine formulation, which has a long-term protection

effect, no side effects, is relatively inexpensive to produce and allows good patient compliance. In active immunization, immune system plays an active role with proliferation of antigen-reactive B cell and T cell, resulting in memory cell formation [2]. The type of adjuvant and the route selected for delivering a vaccine can affect the type of immune response produced, which ultimately governs both the short- and long-term protection against the pathogen. Adjuvants have the ability to selectively modulate the immune response to elicit humoral and/ or cellular immune responses [3].

Mucosal routes of immunization, as well as the skin, are attractive alternatives to parenteral immunization since, with the 'right' system, it is possible to stimulate both arms of the immune system and provide both humoral (antibody) and cell-mediated responses (cytotoxic lymphocytes) [4]. Communication between the MALT and distant mucosal surfaces through cell trafficking has been termed the 'common mucosal immune system'. With regard to the BALT, this would seem to be predominantly a case of gut to bronchus movement of cells. It has been suggested that, in view of a relative paucity of immunocompetent tissue in the BALT, a priming of the intestine followed by a booster exposure of antigen in the respiratory tissue could be more effective in inducing mucosal immune responses than immunization of the respiratory tract alone [5].

However, nasal administration of antigen can result in a better level of IgA in the intestine than oral administration. Such an effect may be due to a difficulty in delivering suitably large quantities of antigen to the correct region(s) of the gut due to dilution effects and to the degradation of sensitive antigenic structures in the acid environment of the stomach. Nasal and (to a lesser extent) pulmonary administration of antigen is an efficient process where it should be possible to administer a dose of vaccine to a preferred site.

Different polymers are being investigated for the use of microparticles preparation. In selecting a polymer, it should be biodegradable, non-toxic, heat-stable, and tend to alter the antigen release rate. In particular, poly-lactic-co-glycolic acid (PLGA) has received tremendous interest for the development of controlled drug delivery systems due to its excellent biocompatibility and biodegradability [6]. These polymers degrade *in vivo* to form non-toxic lactic and glycolic acids and enable the rate of antigen release to be altered through varying the poly-lactide to glycolide ratios [7]. Furthermore, these polymers have the advantage of already having been used in humans and also an FDA-approved polymer.

Integrity of protein structure during encapsulation alone is not sufficient to develop successful sustained release formulations. Proteins however must retain its native active form even after release from the system. Acidity commonly develops in PLGA microspheres because of accumulation of acidic degradation products upon poly-ester hydrolysis, which lead to decline in pH that subsequently may cause irreversible inactivation of encapsulated proteins. In the present study, PLGA microspheres bearing antigen was developed and antigen in the formulation was stabilized by using combinations of protein stabilizers and antacid.

MATERIALS AND METHODS

TT Estimation Using Limes Flocculation (L_f Test)

The total TT antigen was determined by Limes flocculation (L_f test) method as described by [8] and the time taken for the flocculation was noted as K_f. Limes flocculation means (L_f) the tube which contains the optimum concentration of toxin and antitoxin that flocculates first and the corresponding unit of the antitoxin is taken as the L_f value of the toxin.

Estimation of HBsAg

Estimation of HBsAg was carried out by using BCA kit and ELISA method by using AUZYME MONOCLONAL® Kit. Absorbance was measured at 562 nm of each tube against reagent blank for BCA and at 492 nm against a reagent blank for ELISA by double UV-Visible Spectrophotometer. Antibodies against HBsAg were estimated by using AUSAB®EIA Kit. Absorbance of the resultant solutions was measured at492 nm against a reagent blank by double UV-Visible Spectrophotometer [6].

PREPARATION, STABILISAION AND CHARACTERIZATION OF MICROSPHERES

Preparation of HBsAg loaded PLGA Microspheres

A single dose mucosal vaccine for Hepatitis B was developed with HBsAg and formulated with PLGA microspheres by double emulsion method as described in the earlier studies of the author [6]. Formulations PLGA1 to PLGA7 were prepared by changing the concentration of PLGA from 3 to 9% w/v, while the PVA concentration (6% w/v) was kept constant. In the case of formulations PVA1 to PVA10, PLGA concentration was kept constant at 4% w/v while varying the concentration of PVA (Table No. 1). The produced microspheres were subjected to size and morphological studies for optimization. The surface morphology was visualized by scanning electron microscopy (Hitachi Variable Pressure SEM (VP-SEM) S-

3700N, Hitachi High Technologies America, Inc.). The samples for SEM were prepared by sprinkling the microsphere powder on a double adhesive tape that was fixed onto an aluminum slab. The slab was then coated with gold to a thickness of about 300Å using a sputter coater. The samples were then randomly scanned and photographs were taken**. (Fig.1)**

Protein Stabilizers

Different protein stabilizers (Trehalose, Gelatin, Bovine serum albumin (BSA), Human serum albumin (HSA), Sucrose and Hydroxypropyl-β-cyclodextrin (HP-β-CD) at varying concentrations were used in primary emulsion during encapsulation process for TT (model antigen) based PLGA microsphere formulations. Varying concentration of trehalose (0, 0.5, 1.0, 1.5 and 2.0% w/v) was used in the primary emulsion. The respective formulations were coded as PMS-TT-T1, PMS-TT-T2, PMS-TT-T3, PMS-TT-T4 and PMS-TT-T5 respectively. Gelatin was used at 0, 0.2, 0.4, 0.6 and 0.8% w/v concentrations and was coded as PMS-TT-G1, PMS-TT-G2, PMS-TT-G3, PMS-TT-G4 and PMS-TT-G5, respectively. PMS-TT-B1, PMS-TT-B2, PMS-TT-B3, PMS-TT-B4 and PMS-TT-B5 code was given for BSA where in its concentrations were 0, 0.2, 0.4, 0.6 and 0.8% w/v, respectively. Whereas, PMS-TT-H1, PMS-TT-H2, PMS-TT-H3, PMS-TT-H4 and PMS-TT-H5 were coded for HSA and the concentrations were found to be 0, 0.2, 0.4, 0.6 and 0.8% w/v, respectively. Sucrose was used in the concentrations of 0, 0.5, 1.0, 1.5 and 2.0 % w/v and was coded as PMS-TT-S1, PMS-TT-S2, PMS-TT-S3, PMS-TT-S4 and PMS-TT-S5, respectively. HP-β-CD was used in concentrations of 0, 2, 4, 6 and 8% w/v and formulations were labeled as PMS-TT-C1, PMS-TT-C2, PMS-TT-C3, PMS-TT-C4 and PMS-TT-C5, respectively.

In the case of recombinant hepatitis B surface antigen (HBsAg) (candidate antigen) based PLGA microsphere formulations, the same protein stabilizers at similar concentrations [trehalose (0 to 2.0% w/v), gelatin (0 to 0.8% w/v), BSA (0 to 0.8% w/v), HSA (0 to 0.8% w/v), sucrose (0 to 2.0% w/v) and HP-β-CD (0 to 8% w/v) were used as mentioned above and coded as PMS-HB-T1 to PMS-HB-T5, PMS-HB-G1 to PMS-HB-G5, PMS-HB-B1 to PMS-HB-B5, PMS-HB-H1 to PMS-HB-H5, PMS-HB-S1 to PMS-HB-S5 and PMS-HB-C1 to PMS-HB-C5, respectively.

Entrapment efficiency

The loading efficiency of the TT antigen in biodegradable PLGA microspheres was estimated by placing 10 mg of TT-PLGA microspheres in centrifuge tubes and acetonitrile was added to dissolve the polymer [9,10]. The mixture was vortexed, centrifuged and then the supernatant was withdrawn. Complete extraction was ascertained by treating the particles with acetonitrile for 4 times and the residual solvent was removed under vacuum. The remaining solid protein was reconstituted in phosphate buffer saline (PBS, pH 7.4, 0.1M). The total antigen content in the extract was determined by Limes flocculation (L_f test) method as described by [8] and the time taken for the flocculation was noted as K_f. Limes flocculation means (L_f) the tube which contains the optimum concentration of toxin and antitoxin that flocculates first and the corresponding unit of the antitoxin is taken as the L_f value of the toxin. Placebo microspheres were used as control.

The entrapment efficiency of the recombinant hepatitis B antigen in biodegradable PLGA microspheres was determined by dissolving 20 mg of the microspheres in 2 ml of 5% w/v sodium dodecyl sulphate (SDS) in 0.1M sodium hydroxide solution [11]. The amount of antigen was determined by micro bicinchoninic acid assay (BCA) (Genei, Bangalore, India) (n=6). Placebo microspheres were used as control.

In vitro release

In vitro release of TT antigen from PLGA microspheres was carried out in phosphate buffer saline (PBS, pH 7.4). Vials containing 10 mg of TT-PLGA microspheres dispersed in 5 ml of PBS (pH 7.4) were incubated at 37°C on a constant shaking mixer. In the case of HBsAg loaded PLGA microspheres, 50 mg of microspheres dispersed in 5 ml of PBS were incubated at 37°C. One vial was withdrawn at each time-point (day 1, 3, 7, 14, 21, 28, 35 and 42), the contents of vial were centrifuged at 8000 rpm for 10 minutes and the supernatant containing released TT was measured by L_f test (n=6) [8]. In case of HBsAg-PLGA microspheres the released HBsAg was collected and estimated by micro BCA method (n=6) and the same sample was also used to measure *in vitro* antigenicity using an enzyme immunoassay (EIA) kit (AUSZYME®, Abbott Laboratories, USA) (n=6) [12]. Each sample (HBsAg released from the microspheres) was diluted with 0.2% w/v bovine serum albumin (BSA) in PBS (pH 7.4) to get three different concentrations and examined against a linear fitting to the response of control standard samples stored at 4ºC. The *in vitro* antigenicity of HBsAg was evaluated by the ratio of EIA response and protein concentration (EIA/protein). Plain HBsAg, (Shanvac B, Shantha

Biotechnics Ltd., Hyderabad, India) was used as a control standard sample in the same concentrations.

Neutralization & Aggregation of PLGA microspheres

To test whether $Mg(OH)_2$ could neutralize the acidic environment, PBS (pH 7.4) medium containing 5 mg polymer microspheres were incubated at 37ºC for two weeks and pH was measured. The degradation half-life of PLGA microspheres was also determined by a gel permeation chromatography using a Waters 510 pump with a Waters'RI-410 refractive index detector [13]. Molecular weight determination was carried out as follows. Tetrahydrofuran (THF) was the mobile phase at a flow rate of 1ml/min and a temperature of 30°C. Incubated polymer microspheres were dissolved in THF (0.25 % w/v of polymer), filtered, and then injected (20 µl into a set of four µ-Styragel columns (Waters, Bangalore, India) with nominal pore sizes of 10^5, 10^4, 10^3 and 100Å. Average molecular weights were calculated using a series of polystyrene standards as described by (n=6) [14].

Percentage aggregation was also determined as described by [15]. Briefly, incubated polymers were removed from release medium, dried and dissolved in acetone. After centrifugation and removal of the polymer solution, the remaining HBsAg pellet was reconstituted in PBST and incubated (at 37°C) overnight before determining the protein content; this gave a measure of the water-soluble protein encapsulated. Any aggregate was collected by centrifugation and incubated (at 37°C for 30 minutes) in denaturing solvent (PBST, 6M urea, 1mM EDTA); analysis of protein concentration gave the amount of non-covalently bonded HBsAg aggregates. The same procedure was repeated with reducing solvent (10mM dithiothreitol (DTT) in denaturing solvent) to determine the amount of disulfide-bounded aggregates (n=6) [13].

Determination of the Structural Integrity of TT/HBsAg

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed to examine integrity of antigen. TT antigen was extracted by dissolving the microspheres in 2 ml of 5% w/v sodium dodecyl sulphate (SDS) in 0.1M sodium hydroxide solution [11]. The extracted TT antigen solution was loaded onto a 4% stacking gel and subjected to electrophoresis on an 8% separation gel at a constant voltage (150V) until the dye band reached bottom of the gel. The gel was stained with a 0.1% coomassie blue fixative solution, destained using an aqueous solution containing 40% v/v methanol and 10% v/v acetic acid. In case of HBsAg-PLGA microspheres, the extracted HBsAg was concentrated and loaded onto a 3.5% stacking gel and subjected to electrophoresis on a 12% separation gel at 200V (Bio-Rad, USA) until the dye band reached bottom of the gel. Then the gel was stained with silver staining solution and developed using formaldehyde and citric acid solution.

STABILITY STUDIES

The stability of model antigen (tetanus toxoid) and candidate antigen (recombinant hepatitis B surface antigen) within the selected PLGA microspheres (stabilized) core-environment was investigated. Lyophilized TT/HBsAg-PLGA microspheres stabilized with protein stabilizers were incubated at 37°C with RH 60±5% (n=6)[12,13]. One vial was withdrawn at each time-point (day 1, 4, 7, 10, 13, 16, 19). Also, TT/HBsAg-PLGA microsphere based stabilized formulations were incubated at 4˚C for 18 months. Then the antigen was extracted and examined as follows.

Tetanus Toxoid (Model Antigen)

The TT antigen (incubated at 37˚C and 4˚C) was extracted [11] and examined for average particle size, shape, and entrapment efficiency. The entrapment efficiency was determined by L_f test as mentioned earlier.

Hepatitis B Antigen (Candidate Antigen)

The HBsAg (incubated at 37˚C and 4˚C) was examined for average particle size, shape, entrapment efficiency and EIA/protein ratio. The entrapment efficiency (after extracting antigen) was determined by micro BCA assay as mentioned earlier. The *in vitro* antigenicity (EIA/protein ratio) was examined as described earlier using EIA kit (AUSZYME®, Abbott Laboratories, USA). And also the extracted antigen was analyzed by using SDS-PAGE [13] followed by blotting the gels (western blot) onto a cellulose nitrate membrane in glycine/tris transfer buffer at 10 V for one hour (Bio-Rad, USA). The membrane was blocked for one hour in 5% w/v skimmed milk powder in PBS (pH 7.4) containing 0.2% Tween-20 (PBST) and incubated for one hour with polyclonal rabbit anti-HBsAg. After three washings with PBST, the blot was incubated for another hour with goat anti-rabbit IgG conjugated to enzyme. Three washings of PBST were given with an interval of 15 minutes and the bands were visualized. Alum adsorbed HBsAg was used as control.

The intrinsic stability of plain HBsAg (unencapsulated), HBsAg in optimized PLGA microspheres stabilized with trehalose at different pH ranges (pH 3, 4, 5, 6 and 7) was also studied in terms of EIA/protein ratio (*in vitro* antigenicity) at 37ºC (RH 60±5%) for one week [13]. The plain HBsAg incubated at 4˚C was used as positive control.

RESULTS AND DISCUSSION

Estimation of TT& HBsAg

The total TT antigen was determined by Limes flocculation (L_f test) method. Estimation of HBsAg was performed by using BCA method as discussed above. The calibration curve was linearly regressed (Correlation coefficient r2= 0.9991, Intercept = 0.0203, Slope = 0.0011, Equation of line y = 0.0011x + 0.0203) and found to obey the Beer Lambert's law in the concentration range of 12.5 - 1000 µg/ml. The standard errors obtained from the observed values were found to be insignificant. Estimation of HBsAg was also performed by using AUZYME MONOCLONAL® Kit purchased from Abbott Laboratories, USA. Standard curve was prepared, which exhibited a correlation coefficient value of 0.9996, Intercept c =0.0004, Slope m = 0.0169, Equation of line y = 0.0169x + 0.0004 and followed Beer Lambert's law in the concentration range of 1-10 ng/ml. AUSAB®EIA Kit procured from Abbott Laboratories, USA was used for determination of specific antibodies against HBsAg and found to follow Beer Lambert's law in the range of 15-150 mIU/ml with correlation coefficient of 0.9996, Slope = 0.0051, Intercept = 0.0057, Equation of line y = 0.0051x + 0.0057.

Preparation and Characterization of HBsAg loaded PLGA Microspheres

Formulations based on different PLGA concentrations (3-9% w/v) (Table No. 2) PLGA1, PLGA2, PLGA3, PLGA4, PLGA5, PLGA6 and PLGA7 showed an average particles size of 2.85±0.03, 5.16±0.12, 12.01±0.03, 18.12±0.41, 24.13±0.21, 28.91±0.11 and 36.18±0.40 µm, respectively (Table 3.4). A 3-4% w/v PLGA polymer solution was recorded to be ideal for making small microspheres (<10 µm), whereas 5-9% w/v PLGA polymer solution was required to make >10 µm sized microspheres. The 4% w/v PLGA solution (PLGA2) was used in further experiments as an optimum concentration, since 4% w/v concentration provided more homogenously dispersed size range (1-10 µm) than a 3% w/v concentration. Similarly, the concentration of PVA was selected based on the required particle size ranges (1-10 µm). Formulations based on different PVA concentrations (2-11% w/v) (Table No. 3) PVA1, PVA2, PVA3, PVA4, PVA5, PVA6, PVA7, PVA8, PVA9 and PVA10 resulted into an average particle size of 40.07±0.21, 30.12±0.72, 24.96±0.72, 21.80±0.12, 20.01±0.03, 16.01±0.30, 13.15±0.61, 11.21±0.14, 4.69±0.12 and 2.10±0.25 µm, respectively. The 10-11% w/v PVA concentration was thus considered as an ideal for making small microspheres (1-10 µm), whereas 2-9% w/v PVA concentration produced >10 µm sized microspheres. A 10% w/v PVA concentration (PVA9) was therefore selected in further experiments since it provided more homogeneously dispersed size range (1-10 µm) than 11% w/v PVA concentration. In conclusion, 4% w/v PLGA concentration and 10% w/v PVA concentration were optimum determinants in order to get 1-10 µm sized microspheres.

Optimization of PLGA concentration was concluded on the basis of required average particle size (1-10 µm) by microscopic evaluation (optical microscope). The HBsAg containing PLGA microspheres were found to be smooth surfaced without any cracks and pores. This was found by variable Pressure scanning electron microscopy as discussed above. The optimized HBsAg loaded micr4ospheres were found to be ideal as per the expectation based on the morphological studies.

Entrapment Efficiency

The multiple dosage delivery of vaccines and its disadvantages should be overcome by the controlled release vaccine delivery systems. Controlled delivery of a desired antigen over a period of 1-3 months could be achieved using PLGA microspheres. In spite of these merits, the applicability of protein delivery using biodegradable polymers is limited so far. This is primarily due to protein inactivation during the encapsulation process, low encapsulation efficiency and instability. The entrapment efficiency of the microspheres PLGA 1 to PLGA 7 and PVA 1 to PVA 10 before stabilization is studied and tabulated. (Table No. 4)

In the present study, antigen(s) in the PLGA based formulations were stabilized using various protein stabilizers at different concentrations as discussed above which ameliorated the antigen stability and encapsulation efficiency **(Table 5, 6, 7 &8).**

The entrapment efficiency of trehalose stabilized formulations PMS-TT-T1, PMS-TT-T2, PMS-TT-T3, PMS-TT-T4 and PMS-TT-T5 was 37.5, 50.0, 65.0, 85.0 and 90.0%, respectively, while 37.5, 85.0, 85.0, 90.0 and 90.0% entrapment efficiency was recorded for formulations stabilized with gelatin *i.e.*, PMS-TT-G1, PMS-TT-G2, PMS-TT-G3, PMS-TT-G4 and PMS-TT-G5, respectively.

The TT payload of optimized PLGA microspheres stabilized with HP-β-CD *i.e.*, PMS-TT-C1, PMS-TT-C2, PMS-TT-C3, PMS-TT-C4 and PMS-TT-C5 was 37.5, 37.5, 65.0, 80.0 and 75.0%, respectively. Whereas, the entrapment efficiency in case of PLGA microspheres stabilized with BSA, HSA and Sucrose was found out to be relatively lower (65-70%) as compared to Trehalose, Gelatin and HP-β-CD stabilized PLGA microsphere formulations (80-90%).

In-Vitro release

PLGA polymers are soluble in only a limited range of organic solvents and are almost practically insoluble in water. The most commonly used solvent for PLGA is dichloromethane (DCM), because DCM evaporates more rapidly than other solvents such as ethyl acetate or acetone. Therefore, DCM was selected to dissolve the PLGA polymer. Moreover, polymer solubility has also been limited in case of ethyl acetate and acetone. The *in-vitro* release of the microspheres stabilized with protein stabilizers found to be good for the batch PMS-TT-T and PMS-HB-T on comparison with the other batches. (Table No. 9 & 10). EIA/Protein ratio of PMS with HBsAg & Trehalose was studied and reported in Table No. 11 for 42 day *In-Vitro* release study

Neutralization with antacid

In order to prevent the pH drop, $Mg(OH)_2$ was incorporated at different concentrations (0.5, 1.0, 1.5 and 2.0% w/v) into the microspheres, which could prevent structural losses and aggregation of protein. To confirm the neutralization of acidic environment by $Mg(OH)_2$, the percentage aggregation, PLGA degradation and pH of release medium were examined (Table 11). Higher concentrations of $Mg(OH)_2$ (2.0%) showed significant difference ($p<0.05$) in percent aggregation, PLGA degradation and pH of the medium when compared to formulation without $Mg(OH)_2$.

ANTIGEN INTEGRITY

From the above results, trehalose was found to be ideal protein stabilizer for both TT and HBsAg. The structural integrity of the antigen stabilized with trehalose was further confirmed by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) [11]. Antigen was extracted by dissolving the microspheres and the extracted antigen was concentrated and loaded onto a stacking gel and subjected to electrophoresis on a separation gel.

Tetanus toxoid

The antigen integrity of model antigen (TT) before and after encapsulation was examined by SDS-PAGE analysis followed by Coomassie brilliant staining. The antigen integrity of TT within microspheres containing trehalose was intact as shown by SDS-PAGE of the antigen before and after microencapsulation (Fig. 2). But the antigen integrity of the TT without trehalose seemed as if altered after microencapsulation, which might be because of unfolding or aggregation of the antigen at the W/O interface. These results provided a concrete evidence that trehalose plays a role in preventing the inactivation of TT antigen during the process of encapsulation [16,17].

RECOMBINANT HEPATITIS B ANTIGEN

Structural integrity of the candidate antigen (HBsAg) was confirmed by SDS-PAGE and IEF techniques. SDS-PAGE analysis followed by silver staining (Fig. 3) revealed identical bands for the native and entrapped antigen with protein stabilizer (trehalose), but the antigen integrity of the HBsAg without the protein stabilizer was altered after microencapsulation [13], which may be due to antigen unfolding or aggregation at the W/O interface. These results provided convincing evidence that protein stabilizer (trehalose) has positive role in preventing the inactivation of antigen during the process of encapsulation.

IEF analysis is one of the best techniques to test protein degradation–deamidation of the encapsulated and released antigen [15]. Hence, any potential structural alteration of stabilized HBsAg was further confirmed by IEF analysis. Results suggested that the isoelectric point of HBsAg (IEF 5.2 - 5.5) remained same for the native; alum adsorbed HBsAg and encapsulated HBsAg along with stabilizer [13].

Stability Studies

It is important to confirm the stability and activity of proteins during their storage of the optimized microsphere formulations. Hence, stability studies were performed at 37°C (RH 60±5%). The size distribution analysis suggested

that the particle size of individual microsphere was remained same in the range of 1-10 µm. The surface of these microspheres incubated at 37˚C (RH 60±5%) (On day 16) as observed under scanning electron microscope (SEM) was free from any pores or cracks and remained unaltered.

The entrapment efficiency (TT based formulation *i.e.*, model antigen) of trehalose (PMS-TT-T4), gelatin (PMS-TT-G2) and HP-β-CD (PMS-TT-C4) stabilized formulations was 80, 80 and 75, respectively at temperature 37˚C with RH 60±5% on day 16. However, the concentration of retained antigen as estimated after 19 days was dropped to 60, 55 and 50 for TT formulations stabilized with trehalose, gelatin and HP-β-CD, respectively. These results suggested that percent antigen retained in the case of PLGA stabilized TT formulations remained same for 16 days and significant drop was observed after 19 days. In case of HBsAg loaded PLGA formulations stabilized with trehalose (PMS-HB-T4), bovine serum albumin (PMS-HB-B4) and HP-β-CD (PMS-HB-C4) the percent residual antigen estimated was found to be 80.3±1.6, 73.1±3.1, and 74.6±1.3, respectively at 37˚C (RH 60±5%) on day 16 and was further dropped below 60% (<60) after 19 days. These results suggested that HBsAg stabilized formulations were stable at 37˚C (RH 60±5%) for 16 days.

Whereas, the stabilized TT and HBsAg based formulations maintained its entrapment efficiency for 18 months when stored at 4˚C and no significant change in entrapment efficiency was observed upto 18 months. The results of these studies suggested that stabilized TT and HBsAg based PLGA formulations were stable for 16 days at 37˚C with RH 60±5% and significant drop was recorded after 19 days. However, TT/HBsAg-PLGA microspheres stored at 4°C were stable for 18 months.

The *in vitro* immunogenicity of encapsulated HBsAg was evaluated by EIA assay and the EIA/protein ratio of HBsAg based PLGA formulations stabilized with trehalose (PMS-HB-T4), bovine serum albumin (PMS-HB-B4) and HP-β-CD (PMS-HB-C4) incubated at 37˚C (RH 60±5%) was found to be 0.9±0.2, 0.8±0.2 and 0.8±0.1, respectively. The alum adsorbed HBsAg was used as a control and exhibited an EIA/protein ratio of 0.9±0.2 (Day 16). Whereas, the EIA protein ratio was dropped to 0.7±0.2, 0.5±0.08, 0.5±0.1 and 0.5±0.2 for PMS-HB-T4, PMS-HB-B4, PMS-HB-C4 and alum adsorbed HBsAg after 19 days at 37˚C (RH 60±5%). The EIA/protein ratio moreover remained the same for 18 months when the formulations were stored at 4˚C, *i.e.*, no significant changes were observed on its EIA/protein ratio upto 18 months. In conclusion, the *in vitro* antigenicity of alum adsorbed HBsAg (control) and HBsAg-PLGA microspheres stabilized with protein stabilizer (trehalose) was found to be comparable for 16 days and suggested that HBsAg was successfully encapsulated and remained stable at 37°C (RH 60±5%) for 16 days. (Table No.13)

The intrinsic antigenicity of plain HBsAg (unencapsulated), HBsAg in PLGA microspheres at different pH (3-7) was also studied at 37ºC for one week. These results showed that HBsAg lost ~30-50% of *in vitro* antigenicity (EIA/Protein), when the pH was decreased to below 5.0 (pH<5) and remained ~100% active in the pH range of 5-7. Whereas, HBsAg in PLGA microspheres gave an EIA/protein ratio value of 1.0±0.1 (~100% active) in the pH range 3-7 (n=6). The effects of pH (3-7) on *in vitro* antigenicity of each formulations was statistically analyzed (GraphPad, In Stat, USA) and significant ($p<0.05$) increase in i*n-vitro* antigenicity was found in the pH range of 5-7 when compared at pH<5. Plain HBsAg stored at 4ºC (unencapsulated and no 37ºC incubation) was used as control. As pH inside PLGA microspheres was believed to be below 5. $Mg(OH)_2$ was co-incorporated into the PLGA microspheres, which neutralized the acidity during degradation of the polymer and also maintained the pH values between 5-7, in which the antigen was more stable (~100 active) ($p<0.05$). (Table No.14).

The confirmation and activity of HBsAg was also examined by western blot analysis. The encapsulated antigen was extracted from the PLGA microspheres and concentrated using amicon ultrafiltration before being used for the western blot. The immunoreactivity of encapsulated HBsAg was estimated to be unaltered upto 16 days as observed from western blot analysis **(Photograph 4.2).**

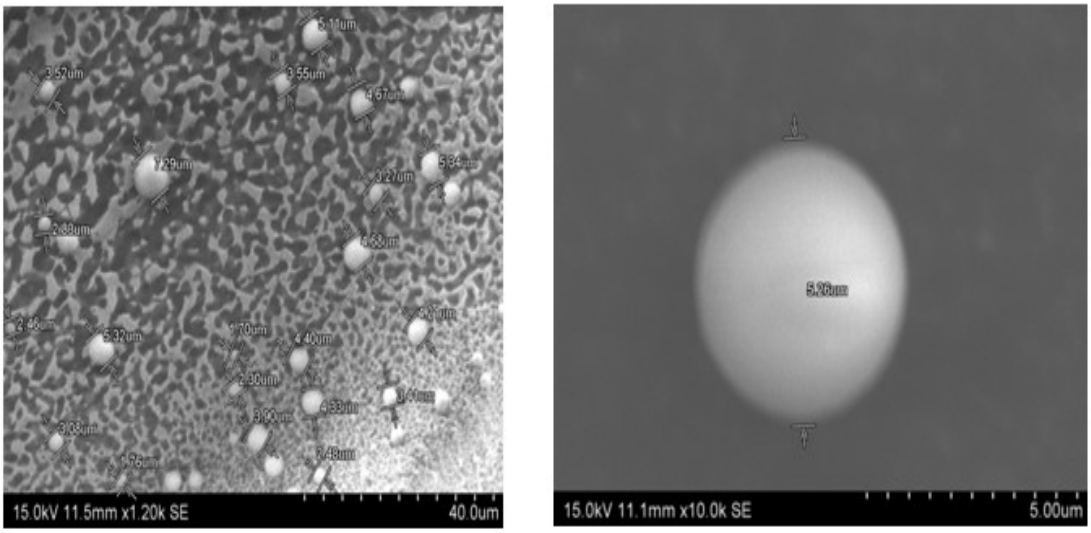

Fig.1: SEM Photographs of HBsAg loaded PLGA microspheres

Table No. 1. Optimization of polymer concentration

S. No.	Formulation code	PLGA concentration (% w/v)	PVA concentration (% w/v)
1	PLGA1	3	6
2	PLGA2	4	6
3	PLGA3	5	6
4	PLGA4	6	6
5	PLGA5	7	6
6	PLGA6	8	6
7	PLGA7	9	6
8	PVA1	4	2
9	PVA2	4	3
10	PVA3	4	4
11	PVA4	4	5
12	PVA5	4	6
13	PVA6	4	7
14	PVA7	4	8
15	PVA8	4	9
16	PVA9	4	10
17	PVA10	4	11

Table No. 2: Average particle size of PLGA based formulations

S. No.	Formulation Code	Average size (µm)*
1.	$PLGA_1$	2.95±0.06
2.	$PLGA_2$	5.86±0.22
3.	$PLGA_3$	14.93±0.08
4.	$PLGA_4$	19.22±0.31
5.	$PLGA_5$	25.15±0.07
6.	$PLGA_6$	27.82±0.31
7.	$PLGA_7$	37.18±0.30

*All values are expressed as mean ± S.D. (n=6)

Table No. 3: Average particle size of PVA based formulations

S. No.	Formulation Code	Average size (μm)*
1.	PVA_1	41.27±0.24
2.	PVA_2	31.12±0.52
3.	PVA_3	23.98±0.62
4.	PVA_4	21.83±0.17
5.	PVA_5	19.01±0.13
6.	PVA_6	15.01±0.40
7.	PVA_7	13.18±0.43
8.	PVA_8	11.31±0.12
9.	PVA_9	4.72±0.16
10.	PVA_{10}	2.56±0.24

*All values are expressed as mean ± S.D. (n=6)

Table No. 4: Entrapment efficiency of Microspheres

S. No.	Formulation Code	% Entrapment efficiency*
1	PLGA1	28.36±3.4
2	PLGA2	30.56±2.0
3	PLGA3	30.88±2.4
4	PLGA4	31.28±3.0
5	PLGA5	29.24±1.8
6	PLGA6	28.84±2.1
7	PLGA7	29.48±3.1
8	PVA1	29.34±2.8
9	PVA2	28.56±4.2
10	PVA3	30.35±5.2
11	PVA4	32.42±2.8
12	PVA5	34.54±2.4
13	PVA6	33.46±4.2
14	PVA7	33.56±2.6
15	PVA8	34.46±18
16	PVA9	32.13±2.8
17	PVA10	31.87±2.3

Table No. 5. Entrapment efficiency of PMS-TT with Trehalose, Gelatin, BSA as protein stabilizers

S.No	Parameter	TT with Trehalose					TT with Gelatin					TT with BSA				
		T_1	T_2	T_3	T_4	T_5	G_1	G_2	G_3	G_4	G_5	B_1	B_2	B_3	B_4	B_5
1	Concentration (% w/v)	0.0	0.5	1.0	1.5	2.0	0.0	0.2	0.4	0.6	0.8	0.0	0.2	0.4	0.6	0.8
2	First flocculated tube (ff)	1	2	5	9	10	1	9	9	10	10	1	1	2	5	4
3	Flocculation time (K_f) (min)	25	25	25	20	20	25	15	15	15	15	25	25	25	25	25
4	% Entrapment efficiency*	38	50	65	85	90	38	85	85	90	90	38	38	50	65	65

Table No. 6. Entrapment efficiency of PMS-TT with HSA, Sucrose, HP-β-CD as protein stabilizers

S.No	Parameter	TT with HSA					TT with Sucrose					TT with HP-β-CD				
		H_1	H_2	H_3	H_4	H_5	S_1	S_2	S_3	S_4	S_5	C_1	C_2	C_3	C_4	C_5
1	Conc. (% w/v)	0.0	0.2	0.4	0.6	0.8	0.0	0.5	1.0	1.5	2.0	0.0	2.0	4.0	6.0	8.0
2	First flocculated tube (ff)	1	1	2	3	2	1	3	5	6	5	1	1	5	8	7
3	Flocculation time (K_f) (min)	25	25	25	25	25	25	25	25	25	25	25	25	23	22	22
4	% Entrapment efficiency*	38	38	50	55	50	38	55	65	70	65	38	38	65	80	75

Table No. 7. Percentage entrapment efficiency of PMS-HB with Trehalose, Gelatin, BSA as protein stabilizers

S.No	Parameter	HBsAg with Trehalose					HBsAg with Gelatin					HBsAg with BSA				
		T_6	T_7	T_8	T_9	T_{10}	G_6	G_7	G_8	G_9	G_{10}	B_6	B_7	B_8	B_9	B_{10}
1	Conc. (% w/v)	0.0	0.5	1.0	1.5	2.0	0.0	0.2	0.4	0.6	0.8	0.0	0.2	0.4	0.6	0.8
4	% Entrapment efficiency*	31	43	68	83	85	30	56	61	63	63	30	48	59	74	76

Table No. 8. Percentage entrapment efficiency of PMS-HB with HAS, Sucrose, HP-β-CD as protein stabilizers

S. No	Parameter	HBsAg with HSA					HBsAg with Sucrose					HBsAg with HP-β-CD				
		H_6	H_7	H_8	H_9	H_{10}	S_6	S_7	S_8	S_9	S_{10}	C_6	C_7	C_8	C_9	C_{10}
1	Conc.(% w/v)	0.0	0.2	0.4	0.6	0.8	0.0	0.5	1.0	1.5	2.0	0.0	2.0	4.0	6.0	8.0
4	% Entrapment efficiency*	30	45	58	65	69	30	42	52	65	64	30	41	62	78	80

Table No. 9. *In-Vitro* release of PMS with TT & Trehalose

S.No	Formulation	PMS with TT & Trehalose % Cumulative release* (Time in days)							
		1	3	7	14	21	28	35	42
1	PMS-TT-T_1	5.5±0.1	5.5±0.1	5.5±0.1	5.5±0.1	5.5±0.1	5.5±0.1	5.5±0.1	5.5±0.1
2	PMS-TT-T_2	10.0±0.5	18.0±0.5	26.0±1.2	40.2±2.1	42.5±2.3	48.0±2.6	52.0±3.1	52.0±2.7
3	PMS-TT-T_3	12.0±0.6	20.0±0.2	38.0±0.7	53.0±0.9	60.0±1.4	68.0±1.6	70.0±2.9	70.0±2.5
4	PMS-TT-T_4	15.0±0.3	30.0±1.8	58.0±2.1	68.8±1.9	75.0±3.1	75.0±3.1	75.0±2.0	75.0±2.1
5	PMS-TT-T_5	22.5±2.1	70.0±3.0	80.0±2.5	80.0±2.1	80.0±1.9	80.0±1.6	80.0±2.8	80.0±1.1

*All values are expressed as mean ± S.D. (n=6)

Table No. 10. *In-Vitro* release of PMS with HBsAg & Trehalose

S.No	Formulation	PMS with HBsAg & Trehalose % Cumulative release* (Time in days)							
		1	3	7	14	21	28	35	42
1	PMS-HB-T_1	5.5±0.6	9.8±0.62	12.5±0.3	15.2±0.3	15.3±0.8	15.6±0.6	15.6±0.4	15.6±1.2
2	PMS-HB-T_2	8.6±0.4	15.5±1.1	31.1±1.1	40.0±2.1	41.3±2.1	44.3±2.1	44.6±2.1	44.8±0.1
3	PMS-HB-T_3	10.0±0.8	15.0±0.9	30.2±2.9	51.0±2.6	62.0±2.8	68.0±2.9	68.5±3.1	68.5±1.1
4	PMS-HB-T_4	11.1±0.9	22.2±1.2	37.7±2.3	62.2±3.5	75.5±3.7	91.1±4.1	91.3±3.5	91.4±4.4
5	PMS-HB-T_5	22.5±3.1	80.0±2.7	92.0±2.2	95.2±3.8	95.2±3.2	95.2±4.0	95.3±3.6	95.3±3.1

*All values are expressed as mean ± S.D. (n=6)

Table No. 11. EIA/Protein ratio of PMS with HBsAg & Trehalose

S.No	Formulation	PMS with HBsAg & Trehalose Ratio of the EIA response to protein concentration* (Time in days)							
		1	3	7	14	21	28	35	42
1	PMS-HB-T_1	0.1±0.01	0.1±0.03	0.2±0.03	0.2±0.05	0.2±0.05	0.3±0.05	0.3±0.04	0.3±0.03
2	PMS-HB-T_2	0.1±0.03	0.1±0.01	0.2±0.01	0.3±0.04	0.3±0.02	0.4±0.01	0.5±0.02	0.5±0.06
3	PMS-HB-T_3	0.1±0.02	0.2±0.01	0.3±0.02	0.4±0.02	0.4±0.03	0.5±0.04	0.8±0.02	0.8±0.06
4	PMS-HB-T_4	0.4±0.01	0.4±0.01	0.5±0.01	0.6±0.03	0.8±0.02	1.0±0.01	1.0±0.04	0.9±0.04
5	PMS-HB-T_5	0.5±0.02	0.9±0.04	0.9±0.06	1.0±0.05	0.8±0.03	0.8±0.05	0.7±0.05	0.7±0.06

*All values are expressed as mean ± S.D. (n=6)

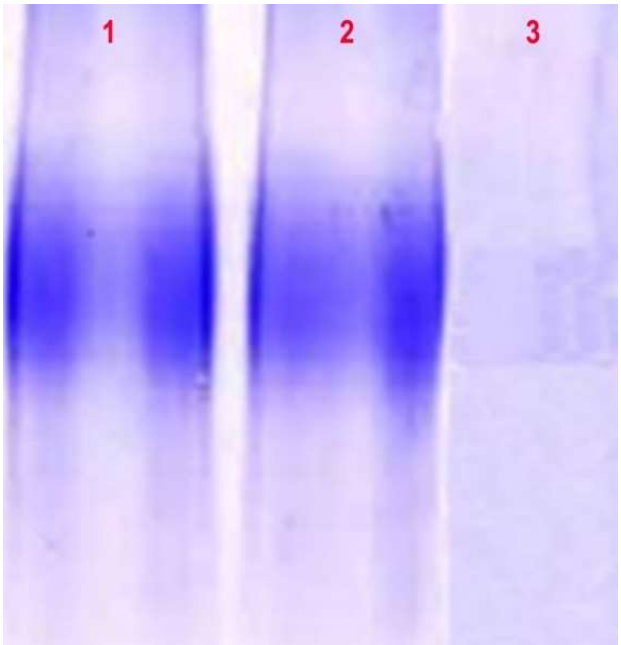

Fig.2 :SDS-PAGE Analysis of TT encapsulated PLGA Microspheres
Lane 1. Standard TT antigen, Lane 2. PMS-TT with Trehalose, Lane 3. PMS

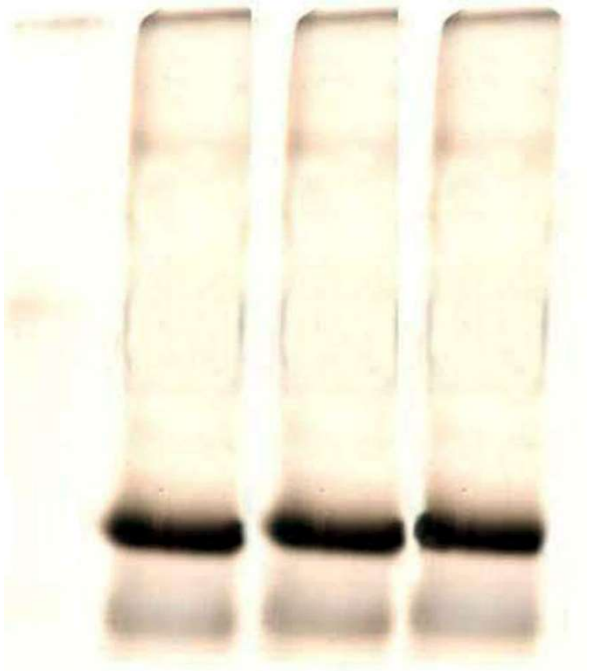

Fig.3: SDS-PAGE Analysis of HBsAg encapsulated PLGA Microspheres
Lane 1. Distilled Water, Lane 2. PMS-HB without Trehalose,
Lane 3. PMS-HB with Trehalose Lane 4. Plain HBsAg

Table No. 12. Neutralization effect of $Mg(OH)_2$ on HBsAg-PLGA microspheres

Property	Concentration of $Mg(OH)_2$ (w/v)				
	0%	0.5%	1.0%	1.5%	2.0%
Aggregation (%)[a]	61±6	48±3	32±4	12±2	1.5±0.5
PLGA degradation $t_{½}$ (days)[b]	12	16	21	25	30
pH of the Medium[c]	3.1	4.2	5.5	6.4	7.0

a. *HBsAg was extracted from microspheres (5mg) after incubation in PBS (pH 7.4) at 37ºC for two weeks (mean±S.E.M., n=6).*
b. *$t_{1/2}$ is the time when the PLGA Mr was reduced to half of the original Mr (determined by GPC) during incubation in PBS (pH 7.4) at 37ºC (n=6).*
c. *PBS (pH 7.4) medium containing 5 mg polymer microspheres after incubation at 37ºC for two weeks (n=6).*

Table No. 13. Percentage entrapment efficiency of PMS-HB-T during Stability studies at 37ºC (RH 60±5%)

S. No.	Formulation	Percentage Entrapment Efficiency*						
		Day 1	Day 4	Day 7	Day 10	Day 13	Day 16	Day 19
1.	PMS-HB-T_1	57.2±2.1	57.1±1.3	56.8±2.3	55.3±2.3	55.1±1.3	50.2±2.7	38.2±2.6
2.	PMS-HB-T_2	74.2±3.5	74.2±3.1	73.9±1.1	73.1±1.8	73.2±1.3	73.1±3.1	41.0±2.1
3.	PMS-HB-T_3	77.1±2.1	76.9±3.1	76.9±2.9	75.3±2.1	75.1±2.3	74.6±1.3	45.0±2.3
4	PMS-HB-T_4	82.5±2.5	82.4±3.2	81.6±0.9	81.5±0.9	81.5±1.9	80.3±1.6	59.3±2.1
5	PMS-HB-T_5	81.1±2.3	80.9±2.1	80.6±2.3	80.3±1.1	79.1±2.2	78.0±1.8	45.0±2.4

*All values are expressed as mean ± S.D. (n=6)

Table No. 14. Percentage entrapment efficiency of PMS-HB-T during Stability studies at 4ºC.

S. No.	Formulation	Percentage Entrapment Efficiency*							
		1 month	2 month	3 month	6 month	9 month	12 month	15 month	18 month
1.	PMS-HB-T_1	57.2±3.1	57.1±2.3	56.9±1.3	56.3±2.6	56.1±1.2	55.9±1.7	58.2±1.6	51.2±2.5
2.	PMS-HB-T_2	74.1±2.5	74.0±2.1	73.8±2.1	72.8±1.3	70.2±1.8	63.1±3.3	51.0±3.1	43.0±2.1
3.	PMS-HB-T_3	78.1±2.2	77.8±2.1	77.6±2.7	75.2±1.1	75.1±1.3	73.6±1.3	72.1±1.3	62.2±1.6
4	PMS-HB-T_4	82.3±2.7	82.2±2.1	82.2±3.1	82.1±2.8	80.9±1.8	80.6±1.8	80.1±1.8	77.6±2.4
5	PMS-HB-T_5	81.4±2.3	81.2±2.1	81.1±2.3	80.5±1.1	80.1±2.2	79.0±1.8	78.2±2.4	69.3±2.1

*All values are expressed as mean ± S.D. (n=6)

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