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Research Article

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Effect of stabilizers on stabilization of HBSAG loaded PLGA microparticles

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ABSTRACT

The aim of this study was to prepare poly(lactic-co-glycolic acid) (PLGA) microspheres containing hepatitis B surface antigen (HBsAg) using different stabilizer such as Sucrose, Trehalose, Bovine serum albumin (BSA), Human serum albumin (HSA), Hydroxypropyl-β-Cyclodextrin (HP-β-CD) and combination of Trehalose and HP-β-CD. Suitable stabilizer is required in the PLGA based formulation containing HBsAg to protect the HBsAg antigenicity loss due to usage of organic solvents, release of acids from PLGA and lyophilization. The developed formulations were characterized for entrapment efficiency, antigen structural integrity, Invitro release study, surface morphology and particle size. The entrapment efficiency observed more with trehalose, HP-β-CD and its combination. There is no major structural antigen integrity by SDS-PAGE for formulations with different stabilizers. The in vitro release test showed that combination of Trehalose and HP-β-CD in both primary and secondary emulsion shows improved antigen recovery to >90% on 42 days release as compared to other formulation. Based on these findings, the HBsA with trehalose and HP-β-CD loaded PLGA microspheres could be an effective carrier delivery system for HBsAg.

Keywords: **S**tabilizers, Trehalose, Hydroxypropyl-β-Cyclodextrin, Hepatitis-B surface antigen, PLGA microparticles

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INTRODUCTION

Hepatitis B infection is one of the serious prevalent infectious diseases continues to be a major public health issue worldwide (1).More than 0.78 Million people die every year due to the hepatitis B virus infection and related ailments (2). Spreading of hepatitis B virus infection can be better controlled by immunization(3). World Health Organization (WHO) has recommended that hepatitis B vaccination routine immunization for all children in worldwide. Currently, aluminum adjuvant based HBsAg vaccines are available for immunization which is safe, efficacious and immunogenic also. However it requires injection followed by a booster dose to achieve long-lasting protective immune responses (4). Most of them are avoiding immunization program due to multiple painful injections and may also lead to spread of infectious agents via contaminated syringes (5). Therefore, it is necessary to explore the alternative safe and effective vaccine delivery system to achieve the simplified immunization program.

Many researches showed that biodegradable and biocompatible polymer such as PLGA based formulations would be capable of reducing the required number of antigen doses for protection to as few as a single shot (6) and used as needle-free based vaccines (7-10). Also several studies reported that HBsAg-loaded PLGA microspheres elicit strong humoral and cellular mediated immune responses (11-13). Even though the applicability of biodegradable polymers for protein delivery is very limited due to protein inactivation during the encapsulation process, low encapsulation efficiency and instability (14-17). The structural integrity of antigen may be lost due to denaturation,

aggregation or precipitation which generally occurs due to the usage of organic solvents during microencapsulation, release of acids from PLGA particles and lyophilization are an important factor that hinders the commercial development of PLGA microsphere based vaccine formulations(18-19).It was necessary to stabilize the protein during both the encapsulation process and the release of protein from the microsphere *In-vivo*. Some of the studies show that usage of stabilizers such as HSA (20), BSA (21, 22), sucrose (23), trehalose (24) and dextran (25) in protein based PLGA formulations. However, the limited literatures are overlooked on the stability of HBsAg during the formulation process.

In the present study, the effect of stabilizer on HBsAg loaded PLGA microsphere was evaluated using the different stabilizers such as Sucrose, Trehalose, BSA, HSA, HP-β-CD and combination of Trehalose and HP-β-CD. HBsAg encapsulated within PLGA microspheres was characterized for entrapment efficiency, antigen structural integrity, *Invitro* release study, Immunogenecity studies, surface morphology and particle size.

EXPERIMENTAL SECTION

2.1 Materials:

Poly(lactic-co-glycolic acid) (PLGA) polymer, sucrose, trehalose, BSA, HSA, HP-β-CD were purchased from Sigma aldrich. Recombinant HBsAg was kindly gifted by one of the private biotechnics pvt ltd at hyderabad. BCA protein estimation kit and protein molecular weight markers were purchased from Genei, Bangalore, India. AUSAB® monoclonal antibody kit was procured from Abbott Laboratories, USA. All other chemicals and reagents were of analytical grade.

2.2 Preparation of PLGA Microparticles:

Surface-modified PLGA microparticles were prepared by a modified double emulsion solvent evaporation process (26). In brief, a primary emulsion (water-in-oil) was formulated by emulsifying aqueous phase containing recombinant HBsAg, different stabilizers in different stabilizers such as sucrose - 2% (F1), Trehalose (T) – 2% (F2), BSA – 0.8% (F3), HAS – 0.8% (F4), HP-β-CD (HP) – 2% (F5), T - 2% + HP – 2% (F6), T – 1.5% + HP – 2% in primary emulsion and T – 0.5% in secondary emulsion (F7) and T – 1.5% + HP – 2% in primary emulsion and T – 0.5% + HP – 2% in secondary emulsion (F8). Followed by addition of 2%(w/v) $Mg(OH)_2$ with 4% w/v PLGA in methylenechloride using a probe sonicator (Soniweld, India) for 1 min. To this water-in-oil emulsion, 10% w/v of aqueous polyvinyl alcohol was added and mixed at high speed with an Ultraturrax T-25 homogenizer (IKA, Germany) for 3 minutes to obtain a W/O/W emulsion. The resultant emulsion was stirred vigorously for 3 h to evaporate the organic phase and to obtain the microparticles, which were collected by centrifugation at 22,000 g and washed twice with distilled water to remove PVA. The microparticles were then subjected to lyophilization. Different stabilizers used in PLGA based microsphere formulation details are captured in table 1. Alum based HBsAg was prepared as per Arash Mahboubi et al method (27) and used as control for Immunogenecity studies.

2.3 Microsphere particle size and protein Loading Efficiency

The mean diameter of the HBsAg loaded microsphere formulation was determined by dynamic light scattering usingCILAS particle size analyzer. Normal saline was used as the dispersion medium for the determination of particle size. The loading efficiency/entrapment efficiency of the antigen in microparticles was determined by dissolving 20 mg of the microparticles in 2 ml of 5% (w/v) sodium dodecyl sulfate (SDS) prepared in 0.1 M sodium hydroxide solution. And the amount of the antigen present in the solution was determined by the bicinchoninic acid assay using the BCA protein estimation kit. The loading efficacy (LE) of HBsAg loaded PLGA microparticles were calculated from the following equations:

$$\text{Loading Efficiency (\%)} = \frac{\text{(Total amount of HBsAg – Free HBsAg)}}{\text{Total amount of HBsAg}} \text{ X } 100$$

2.4 Assessment of Structural Integrity of HBsAg

The structural integrity of HBsAg loaded microsphere was detected by SDS polyacrylamide gel electrophoresis (PAGE) and compared with the native HBsAg and reference markers. First, HBsAg was extracted from microparticles by dissolving in 2 ml of 5% (w/v) SDS in 0.1 M sodium hydroxide solution (28). The extracted antigen was subjected to electrophoresis at 200 V (Bio-Rad, USA) in 3.5% stacking gel followed by 12% separation

gel until the dye band reached the gel bottom. After electrophoresis, the protein bands were stained using Coomassie Blue R-250 followed by de-stained and dried.

2.5 *In Vitro* antigen release test

HBsAg drug release was estimated using *In vitro* dissolution medium. Briefly,40 mg of microparticles was suspended in 5 ml of phosphate buffered saline (PBS; pH 7.4) and kept for shaking (50 rpm) at 37°C. Tween-80 (0.02%, w/v) was added in release media to reduce the adsorption of the released protein, to prevent the particles from clumping and improve their wettability. At suitable time intervals, 1.0 ml of release medium was collected and centrifuged at 22,000 g for 30 min and tested for *Invitro* release of HBsAg. During each withdrawal, 1.0 ml of fresh PBS (pH 7.4) was again added to maintain the sink conditions.

2.6 Immunological study:

Anti-HBsAg antibodies in blood samples were determined by using an enzyme-linked immunoassay (ELISA). The samples were collected from mice for IgG immunological study according to the method reported by Jaganathan et al., (26).In Brief, each well of microtiter plates (Nunc-Immuno Plate® Fb 96 Mexisorp, NUNC) were coated with 100 µl/well of 2 µg/ml HBsAg in carbonate buffer (pH 9.6) and incubated overnight at 5 ±3°C. The plates were washed with three times using PBS-Tween 20 (0.05%, v/v) (PBS-T) and blocked with PBS-BSA (3% w/v) for 2 h at 37°C, followed by washing with PBS-T. The serum/secretion samples were serially diluted with PBS. One hundred microliters of these serially diluted serum and secretion samples were added to the wells of coated ELISA plates. The plates were incubated for 1 h at room temperature and washed three times with PBS-T. One hundred microliters of horse reddish peroxidase labeled goat anti-mouse IgG(1:1,000 dilution, Sigma, USA) antibodies were added to well for the determination of IgG titer, respectively. The plates were kept for 1 h at room temperature and then washing was repeated. One hundred microliters of tetramethylbenzidine (TMB-H_2O_2) solution was added to each well. Color development was stopped after 30 min by adding 50 µl of 1 N H_2SO_4 to each well, and absorbance was taken at 490 nm using a plate reader (Bio-Rad, USA). The end-point titers were expressed as the log reciprocal of the last dilution, which gave the absorbance value above the absorbance of negative control at a wavelength of 490 nm.

2.7 Statistical analysis:

Statistical analysis was performed on the data obtained in the *In vivo* studies by one-way analysis of variance (ANOVA) with Tukey's multiple comparisons post test using GraphPadInStatTM software (GraphPad Software Inc., San Diego, California). A value of $P < 0.05$ was considered to be statistically significant.

RESULTS AND DISCUSSION

3.1 Microsphere particle size and protein loading efficiency

HBsAg loaded PLGA microspheres with different stabilizers were prepared using the modified double emulsion method. In F1 to F6 formulations, different stabilizers such as Sucrose (2%), Trehalose (2%), Bovine serum albumin (BSA) (0.8%), Human serum albumin (HSA) (0.8%), HP-β-CD (2%) and combination of trehalose (2%) and HP-β-CD (2%) incorporated in primary emulsion, whereas F7 (trehalose (1.5%) and HP-β-CD (2%) in primary emulsion, trehalose (0.5%) in secondary emulsion) and in F8 (trehalose (1.5%) and HP-β-CD (2%) in primary emulsion, trehalose (0.5%) and HP-β-CD (1%) in secondary emulsion). The particle characteristics of the HBsAg loaded microsphere with different stabilizer was found between 3 to 5µm (Table 2).The antigen loading efficiency with different stabilizers were found to be comparable(Table 2). Formulation with trehalose alone (F2), combination of trehalose and HP-β-CD in primary emulsion (F6) and combination of trehalose and HP-β-CD in both primary and secondary emulsion (F8) shows antigen loading efficiency with more than 80%.

3.2 Confirmation of the Structural Integrity of the Antigen

During the microparticles formulation use of organic solvents or release of lactic acid and glycolic acid from PLGA may disturbs the native form of antigen such as aggregation or increase in impurity profile. To maintain the stability of antigen in the microsphere, we used different stabilizer in different formulation and $Mg(OH)_2$ as acid neutralizing agent. Structural integrity of the entrapped antigen in microsphere was assessed using SDS-PAGE (Figure 1). The SDS-PAGE analysis revealed that the native antigen as control and antigen released from the formulation using different stabilizer demonstrated identical bands. This confirmed that structural integrity of antigen remains stable during the encapsulation and release process. It was hypothesized that the protein stabilizer could shield their

antigens from the organic solvent via preferential hydration of their surface, thus preventing protein interface exposure to deleterious solvent effects (29-31)

Table-I: HBsAg loaded microsphere using different stabilizer

Formulation code	Stabilizer	Concentration in primary emulsion	Concentration in secondary emulsion
F1	Sucrose	2%	-
F2	Trehalose (T)	2%	-
F3	BSA	0.8%	-
F4	HSA	0.8%	-
F5	HP-β-CD (HP)	2%	-
F6	T + HP	2% + 2%	-
F7	T + HP	1.5% + 2%	0.5% + 0%
F8	T + HP	1.5% + 2%	0.5% + 1%

Table-II: Percentage loading efficiency of HBsAg loaded PLGA microparticle

Formulation code	Particle size	% Loading efficiency
F1	3.49 ± 0.09	64.1±3.8
F2	3.47 ± 0.11	82.3 ± 1.3
F3	4.64 ± 0.03	63.1± 2.1
F4	3.52 ± 0.05	68.2 ± 1.7
F5	3.84 ± 0.15	72.6±1.2
F6	4.82 ± 0.08	80.1± 2.1
F7	4.53 ± 0.13	77.6± 1.3
F8	4.64 ± 0.03	84.7 ± 1.8

Results are expressed as mean ±S.D (n = 6).

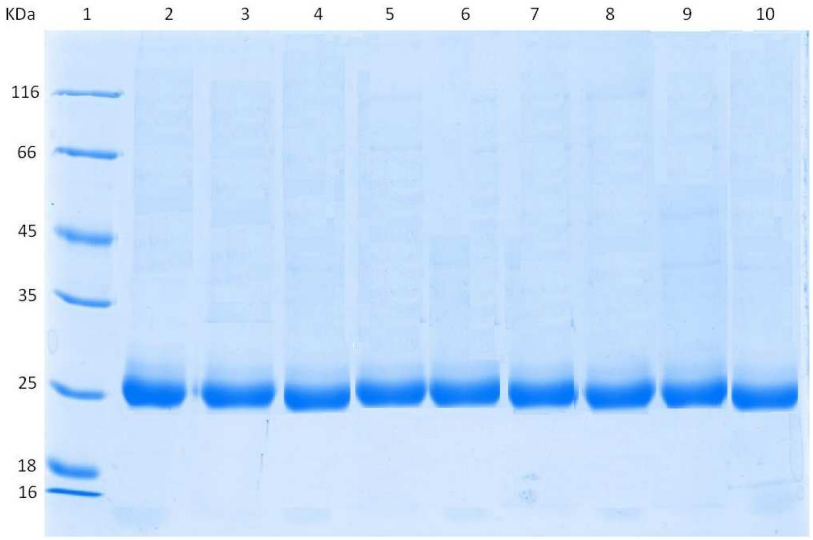

Figure 1: SDS–PAGE analysis: SDS–PAGE showing stability of antigen isolated from formulations. (Lane 1) Marker proteins, (Lane 2) native HBsAg (24 kDa); (Lane 3–10) HBsAg loaded PLGA Microsphere with stabilizers F1-F8 respectively (24 kDa)

3.3*In Vitro* antigen Release profile

In microspheres based formulations, different stabilizers and magnesium hydroxide as neutralizer were incorporated to protect the antigens from the damages due to organic solvent, high shear agitation and low pH environment. *In vitro* release of HBsAg from the microparticles was determined in PBS (pH 7.4). However, the release profiles of PLGA microsphere with different stabilizer exhibited a similar biphasic pattern i.e. an initial burst release, in which alarge amount of HBsAg was released within one day followed by a sustained release of HBsAg over sixty days (Figure 2). Formulation with trehalose alone (F2) and combination of trehalose and HP-β-CD in both primary and secondary emulsion (F8) released above 90% in 42 days as compared to other formulations released 44 to 79% within the same period.

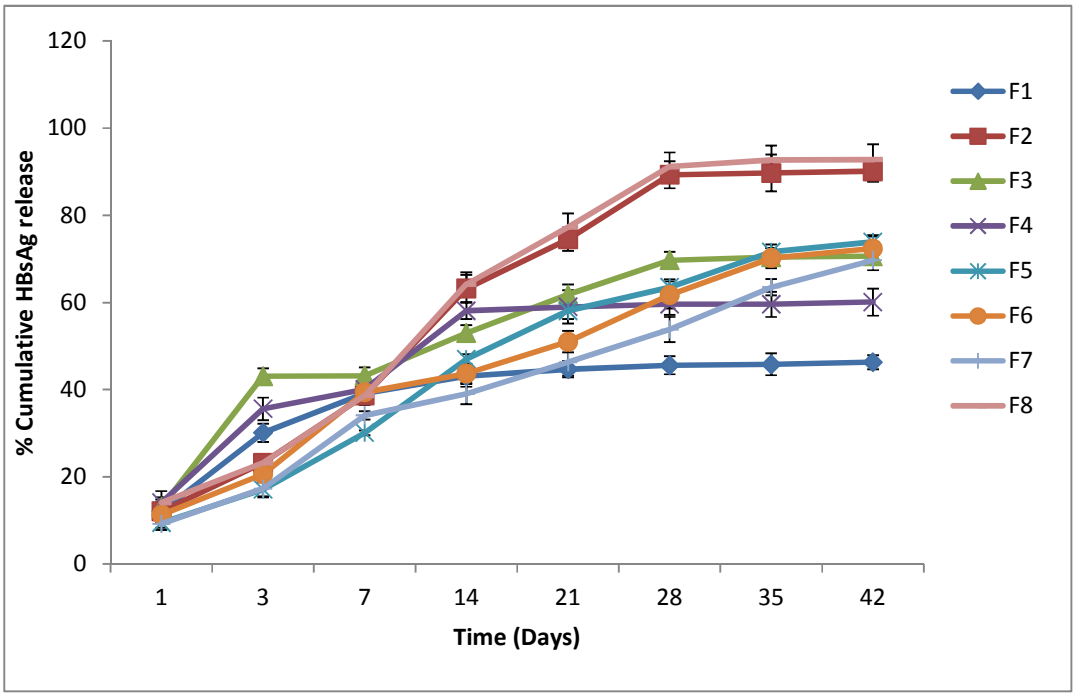

Figure2:*In vitro* release study of % antigen release with respect to time in PBS (pH 7.4)

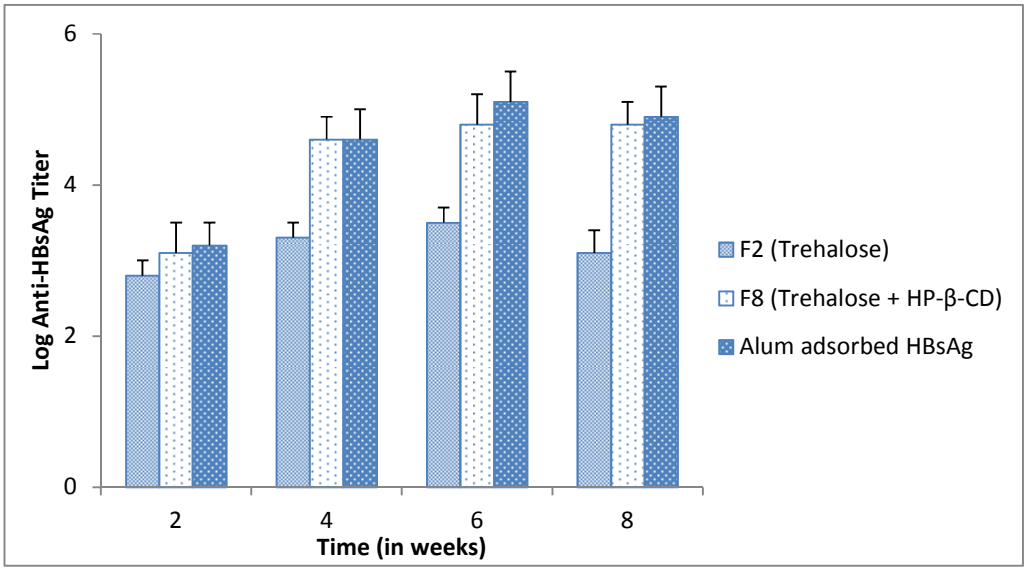

Figure3: Anti-hepatitis B surface antigen IgG titer in serum

3.4 Immunological study:

Based on the encapsulation efficiency, structural integrity of antigen and *In vitro* release profile, formulation with stabilizer such as trehalose alone (F2) and combination of trehalose and HP-β-CD in both primary and secondary emulsion (F8) selected for the immunological studies and alum-based HBsAg was used as a control. The immune effect of the optimized formulations investigated using the different groups of animals were immunized subcutaneously. Serum IgGlevel in blood was determined using ELISA. The results shows that 60th day (i.e., 90th day after primary dose) of serum antibody titres indicated that a single injection of PLGA formulated HBsAg

vaccine with combination of trehalose and HP-β-CD in both primary and secondary emulsion (protein stabilizer) produced almost equivalent immune response when compared with two injections (with booster) of alum adsorbed HBsAg vaccine (Figure 3). Whereas, PLGA formulated HBsAg vaccine with trehalose alone and a single injection of alum adsorbed HBsAg vaccine did not result in any significant IgG antibody titres ($p<0.05$).Based on this study HBsAg-PLGA microspheres stabilized with combination of trehalose and HP-β-CD in both primary and secondary emulsion shows significant IgG level compared to formulation with trehalose alone.

CONCLUSION

The protective effects of different stabilizer on the stability of HBsAg microsphere formulation and its release profile were explored in the study. Formulation containing stabilizers such as trehalose - 2% alone (F2) and combination of trehalose– 1.5% and HP-β-CD– 2% in primary emulsion and trehalose – 0.5% + HP-β-CD – 2% in secondary emulsion (F8) observes higher stabilization effect compared to other formulations based on the encapsulation efficiency, structural integrity of antigen and *In vitro* release profile. The results evidenced that the better immunogenicity was produced with single injection of PLGA microparticles of HBsAg with combination of trehalose and HP-β-CD in both primary and secondary emulsion almost equivalent immune response shows better as compared to single injection of formulation with trehalose alone and single injections of the conventional alum-HBsAg vaccine. And produced equivalent immunogenicity effect when compared with two injections (with booster) of alum adsorbed HBsAg vaccine. Finally on the basis of *In vitro* and *In vivo* studies, it is conclude that HBsAg loaded PLGA microparticles with combination of trehalose and HP-β-CD could be an effective stabilizer to release antigen safely in targeted area.

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