DEVELOPMENT AND CHARACTERIZATION OF HBSAG LOADED PLGA MICROSPHERES FOR MUCO-ADHESIVE DELIVERY SYSTEMS

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Abstract

This work was designed to develop muco-adhesive PLGA microspheres containing HBsAg and to characterize them. The Hepatitis B surface antigen was estimated by using BCA kit and by ELISA methods. Specific antibodies against HBsAg were measured using AUSAB®EIA Kit. Microspheres were prepared by double emulsion evaporation technique. 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 from 2 to 11 % w/v. Surface morphology and size analysis of PLGA microspheres were done by using bright field microscope and by SEM analysis. The average size range of the microspheres varied from 2.56 μm to 41.27 μm. The size of the microspheres was directly proportional to the concentration of PLGA and was inversely proportional to the concentration of PVA. The percentage entrapment efficiency of the HBsAg was studied and was ranged from 28.36 to 34.54 for the formulations. Four microspheres formulations which have been produced within the size range of 1-10 μm have been selected for the *in-vitro* release studies. The *in-vitro* cumulative % release pattern of all the three formulations was very similar except PLGA2. The microspheres will be further improved for the entrapment efficiency and for a better *in-vitro* release profile.

Key words: PLGA Microspheres, Hepatitis B, Muco-adhesive, ELISA and BCA.

Introduction

Microspheres are solid colloidal particles with diameters in the micrometer range, into which antigenic components and/or adjuvant may be entrapped, chemically attached or adsorbed onto the surface[1]. Microspheres exhibit many advantages for vaccine development. They protect entrapped antigen from harsh GIT conditions[2], allow for controlled antigen release thus minimizing the number of doses required for immunization[3] and may allow exogenous antigens to be targeted to endogenous antigen processing pathways for presentation to CTLs[4].

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Different polymers have been investigated for use in microparticles preparation. In selecting a polymer, it should be biodegradable so that it will not require surgical removal following drug/antigen depletion. Furthermore, it should be non-toxic, heat-stable, and allow for alteration of the antigen release rate [3]. A great variety of both natural and synthetic biodegradable polymers such as chitosan, gelatin, polylactic-coglycolic acid, polyalkyl cyanoacrylate, polymethyl methacrylate, polylactic acid and polycaprolactone are used for the preparation of drug loaded microspheres[5-7]. In particular, polylactic-co-glycolic acid (PLGA) has received tremendous interest for the development of controlled drug delivery systems due to its excellent biocompatibility and biodegradability[8]. Several methods, including phase separation or coacervation, emulsification diffusion,

spray-drying, and emulsion-solvent evaporation techniques have been used to obtain PLGA microspheres[9]. With the emulsion solvent evaporation technique, numerous hydrophilic drug substances including proteins and peptides have been encapsulated into PLGA nano- and microparticles using w/o/w double emulsion methods and the mechanism of drug release from these particles has been thoroughly studied[10,11].

Materials and Methods
Standardization
Estimation of HBsAg Using BCA Kit

Bicinchoninic acid (BCA) based method was used for the estimation of HBsAg using protein estimation kit (KT-31, Bangalore Genei Pvt. Ltd., Bangalore). Briefly, BSA standard solutions were prepared with the reagents provided in the kit using PBS (pH 7.4) as diluent. BCA working reagent (BWR) was prepared by mixing 50 parts of reagent A (sodium carbonate, sodium bicarbonate, bicinchoninic acid and sodium tartrate in 0.1M sodium hydroxide) with 1 part of reagent B (copper sulphate solution). 0.2 ml of standard and unknown samples was transferred to labeled test tubes. 0.2 ml of diluent was used as a blank and 2.0 ml of BWR was added to each tube with constant agitation. All the tubes were incubated at 37°C for 30 minutes followed by cooling to room temperature. Absorbance was measured at 562 nm of each tube against reagent blank by double UV-Visible Spectrophotometer (GBC Cintra10, GBC Scientific Equipment, Australia).

Estimation of HBsAg by ELISA

AUZYME MONOCLONAL® Kit purchased from Abbott Laboratories, USA was used for determination of HBsAg. Standard curve was plotted according to the instructions provided by the manufacturer. Recombinant HBsAg in the concentration range of 1-10 ng/ml was used for preparing calibration curve. One positive, one negative control and one reagent blank were prepared. 200 µl of positive control, negative control, double distilled water and different concentrations of HBsAg (1-10 ng/ml) were dispensed into bottom of wells of reaction tray. Followed by addition of 50 µl of anti-HBsAg conjugated to horseradish peroxidase (HRPO) enzyme to each well and tray was gently tapped to mix the contents. One bead coated with antibodies against HBsAg was added to each well and incubated at 40±1°C for 2 hours. Subsequently, liquid was aspirated and beads were thoroughly washed four times with double distilled water. Washed beads were transferred to properly identified assay tubes. Freshly prepared O-Phenylenediamine (OPD) substrate solution (300 µl) was added to each tube and incubated for 30 minutes at room temperature. The reaction was stopped using 1N H_2SO_4. Absorbance of controls and specimen samples was measured at 492 nm against a reagent blank by double UV-Visible Spectrophotometer (GBC Cintra10, GBC Scientific Equipment, Australia).

Estimation of Antibodies against HBsAg

Specific antibodies against HBsAg were measured using AUSAB®EIA Kit procured from Abbott Laboratories, USA. The AUSAB EIA test uses the "Sandwich principle" a solid phase enzyme-linked immunoassay technique for determination of anti-HBs antibodies. The test specimens were serially diluted using specimen dilution buffer (negative human plasma). Serum specimen dilutions, negative, positive controls as well as blanks in 200 µl volume were taken along with human serum standards of known anti-HBs concentration provided with the kit in separate wells. HBsAg coated beads were added to each well and incubated at 40±1°C for 2 hours. 200 µl of biotin-HBsAg and Anti-biotin-HRPO were added into each well and incubated at 40±1°C for 2 hours. Subsequently, the liquid was aspirated and beads were transferred to previously identified assay tubes. 300 µl of freshly prepared OPD substrate solution was added to each tube and incubated for 30 minutes at room temperature. 1N H_2SO_4 was added to each tube to stop the reaction and absorbance of the resultant solutions was measured at 492 nm against a reagent blank.

Preparation of HBsAg loaded PLGA Microspheres

PLGA microspheres (PLGAMS) were prepared by a double emulsion method, aseptically at room temperature, as was reported by Shi *et al.* (2002) with slight modifications[13]. PLGA in methylene chloride was sterilized by membrane filtration (0.22 µm PTFE membrane, Millipore, USA). In brief, 800 µl of

recombinant hepatitis B surface antigen (HBsAg) containing protein stabilizers at different concentrations and $Mg(OH)_2$ was suspended in 10 ml of sterile PLGA in methylene chloride (3 to 9% w/v) and sonicated for 10 seconds at 50W in an ice bath (Soniweld, New Delhi, India). To this water-in-oil emulsion, 40 ml of aqueous PVA (2-11% w/v) was added and mixed at high speed with an Ultraturrax T-25 homogenizer (IKA, Germany) for 10 seconds, to obtain a W/O/W emulsion. The so formed W/O/W emulsion was then poured into 50 ml of 0.3% w/v aqueous PVA under vigorous stirring for one hour. 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 from 2 to 11 % w/v. The microspheres were collected by centrifugation, washed with distilled water and subjected to lyophilization[14] as described below.

The microsphere formulation was filled manually into 5 ml vials and partially closed with rubber stoppers in a sterile working area. Then, the vials were transferred onto the lyophilizer shelves pre-cooled at 4±1˚C. The freezing step was performed at 4±1˚C for 2 hours and followed by -50±2˚C for 3 hours. The primary drying was conducted at −28±1˚C (product temperature) with chamber pressure at 125±2 mTorr for 6 hours and followed by -10±0.2˚C (100±2 mTorr) for 5 hours. The condenser temperature was found to be -90±1˚C. The secondary drying was conducted at 25±1˚C (product temperature) with chamber pressure at 30 mTorr for 5 hours. All the dried samples were stoppered under vacuum at 30 mTorr. The vials were then sealed and placed in a dessicator at 4°C for further studies.

Characterization of Microspheres
Size and Morphology
The prepared microspheres were subjected to light microscope analysis to study the surface morphology[9]. A drop of freshly prepared microspheres was placed on a clean and dried glass slide which was then covered with a cover slip to focus under the 100X magnification. This was carried out to study the morphology, size uniformity, and aggregation or coalescence of the microspheres being prepared.

The surface morphology was visualized by scanning electron microscopy. The samples for SEM were prepared by sprinkling the microsphere powder on a double adhesive tape that was fixed onto an aluminum stab. The stab was then coated with gold to a thickness of about $300A^0$ using a sputter coater. The samples were then randomly scanned and photographs were taken[15].

Estimation of HBsAg Content in PLGA Microspheres
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[16]. The amount of antigen was determined by micro bicinchoninic acid (BCA) assay (Genei, Bangalore, India). Placebo microspheres (without HBsAg) were used as control.

In vitro Release of HBsAg from Microspheres
In vitro release of HBsAg from PLGA microspheres was carried out in phosphate buffer saline (PBS, pH 7.4). Vials containing 50 mg of HBsAg loaded PLGA microspheres dispersed in 5 ml of PBS (pH 7.4) were incubated at 37°C on a constant shaking mixer. 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 HBsAg was collected and estimated by micro BCA method and the same sample was also used to measure *in vitro* antigenicity using an enzyme immunoassay (EIA) kit (AUSZYME®, Abbott Laboratories, USA)[1]. 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 4°C. The *in vitro* antigenicity of HBsAg was evaluated by the ratio of EIA response and protein concentration (EIA/protein). Plain HBsAg (sample of internal reference standard used in Shantha Biotechnics Ltd., Hyderabad, India) was used as a control standard sample in the same concentrations[14].

Table 01: Average size, Percentage entrapment efficiency of HBsAg loaded PLGA Microspheres

S. No.	Formulation Code	PLGA Conc. (% w/v)	PVA Conc. (% w/v)	Average size (μm)*	% Entrapment efficiency*
1.	PLGA1	3	6	2.95±0.06	28.36±3.4
2.	PLGA2	4	6	5.86±0.22	30.56±2.0
3.	PLGA3	5	6	14.93±0.08	30.88±2.4
4.	PLGA4	6	6	19.22±0.31	31.28±3.0
5.	PLGA5	7	6	25.15±0.07	29.24±1.8
6.	PLGA6	8	6	27.82±0.31	28.84±2.1
7.	PLGA7	9	6	37.18±0.30	29.48±3.1
8.	PVA1	4	2	41.27±0.24	29.34±2.8
9.	PVA2	4	3	31.12±0.52	28.56±4.2
10.	PVA3	4	4	23.98±0.62	30.35±5.2
11.	PVA4	4	5	21.83±0.17	32.42±2.8
12.	PVA5	4	6	19.01±0.13	34.54±2.4
13.	PVA6	4	7	15.01±0.40	33.46±4.2
14.	PVA7	4	8	13.18±0.43	33.56±2.6
15.	PVA8	4	9	11.31±0.12	34.46±18
16.	PVA9	4	10	4.72±0.16	32.13±2.8
17.	PVA10	4	11	2.56±0.24	31.87±2.3

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

Table 02: *In- vitro* Release of HBsAg from PLGA Microspheres

S. No.	Formulation Code	% Cumulative release* (Time in days)							
		1	3	7	14	21	28	35	42
1.	PLGA1	5.4±0.6	8.9±0.62	11.9±0.3	16.4±0.3	17.2±0.8	17.6±0.6	17.9±0.4	18.1±1.2
2.	PLGA2	5.6±0.4	9.3±1.1	12.1±1.1	14.0±2.1	16.3±2.1	17.8±2.1	18.6±2.1	19.8±0.1
3.	PVA9	5.2±0.8	8.0±0.9	11.2±2.9	16.0±2.6	17.0±2.8	17.2±2.9	17.5±3.1	17.7±1.1
4.	PVA10	5.2±0.9	8.2±1.2	11.7±2.3	16.2±3.5	17.5±3.7	18.1±4.1	18.3±3.5	18.8±4.4

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

Results and Discussion

Estimation of HBsAg was performed by using BCA method as discussed above. The calibration curve was linearly regressed (Correlation coefficient r^2= 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.

The size of the prepared microspheres was measured by light microscopy-stage micrometer method. The values were tabulated and the average size of each formulation was calculated with standard deviation. The average size range of the microspheres varied from 2.56 μm to 41.27 μm. On increasing the PLGA concentration by keeping the PVA concentration as constant, the size of the microspheres was increasing from 2.95 μm to 37.18 μm. But on increasing the PVA concentration by keeping the PLGA concentration as constant, the size of the microspheres was decreasing from 41.27 μm to 2.56 μm (Table. 1).

The percentage entrapment efficiency was calculated after finding out the amount of HBsAg incorporated in each formulation and the values were tabulated. The

percentage entrapment efficiency was ranged from 28.36 to 34.54 (Table. 1).

There is not much difference in the entrapment efficiency of the various formulations produced. The concentrations of PLGA or PVA were not found to have any effect on entrapment efficiency. The four formulations which have produced the microspheres within the size range of 1-10 μm have been selected for the *in-vitro* release studies. The release profile was interesting to note and the cumulative % release pattern of all the above four formulations were very similar except PLGA2 (Table. 2).

Conclusion

From the above observations it can be concluded that the size of the microspheres is depends on the concentration of PLGA and PVA. On increasing the concentration of PLGA the size of the microspheres also get increased and on increasing the concentration of PVA the size of the microspheres get decreased. The size of the microspheres can be regulated by changing the concentration of PLGA and PVA. The size ranges of the microspheres have not shown any effect on the entrapment efficiency and hence for the regulation of the entrapment efficiency PLGA or PVA cannot be used. Particle size between 1 μm to 10 μm is suitable for the study and four formulations were selected for *in-vitro* release study. Based on the results obtained from the *in-vitro* release study the formulation 2 was selected for further studies. The other factors like particle size distribution and tendency to get aggregation were also comparatively good for the PLGA 2 than the other formulations. Further the microspheres will be investigated to increase its entrapment efficiency with stabilization and surface modification.

Acknowledgement

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