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Development and Characterization of Mucoadhesive HBsAg PLGA Microsphere for Nasal Vaccine Delivery

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ABSTRACT

The purpose of the study was to evaluate the mucoadhesive property of Hepatitis B surface antigen (HBsAg) loaded surface modified poly(lactic-co-glycolic acid) (PLGA) microspheres for nasal vaccine delivery. The surface modification was carried out using coating material such as chitosan, Trimethyl chitosan and N-trimethyl chitosan and N-carboxymethyl chitosan (TMC-CMC). The developed formulations were characterized for surface morphology, particle size, zeta potential, entrapment efficiency, structural integrity, *In vitro* mucoadhesion study and mucin adsorption ability. PLGA microspheres without surface modification demonstrated negative zeta potential, whereas Chitosan and its derivatives coated microspheres showed higher positive zeta potential. Results indicated that combination of TMC-CMC coated microspheres demonstrated substantially higher mucin adsorption and longer time of mucoadhesion when compared to chitosan and TMC coated microspheres and uncoated PLGA microspheres. Both uncoated and coated PLGA microspheres showed initial burst release followed by prolonged release pattern. The immuno-adjuvant ability of various formulations was determined on the basis of specific antibody titer observed in serum and secretions of mice. *In vivo* immunogenicity studies showed increased anti-HBsAg titer with TCC-CMC coated PLGA microspheres as compared to other coated and uncoated PLGA microspheres. To conclude, TCC-CMC coated PLGA microspheres could be a promising carrier targeted delivery for HBsAg in nasal mucosa.

Key-words: Carboxymethyl chitosan, Mucosal immunization, Nasal vaccine delivery, Hepatitis-B surface antigen, PLGA microspheres

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1.0. INTRODUCTION

Hepatitis B surface antigen (HBsAg) is one of the most prevalent infectious diseases worldwide. As per WHO, 240 million people are chronically infected with hepatitis B and more than 0.78 Million people die every year due to the of hepatitis B complications including cirrhosis and liver cancer (1). HBsAg may be transmitted to others through contact with infected blood or other body fluids of an infected person. A Hepatitis B infection is one of the vaccine preventable diseases, so immunization with HBsAg vaccine is the best preventive measure to avoid infection. Currently HBsAg vaccines are available with aluminum-based adjuvant (aluminum hydroxide or aluminum phosphate) and it's recommended to be administered through intramuscular route, but it does not induce mucosal antibodies efficiently (2).

Mucosal route of vaccine delivery is one of the best alternative to conventional multiple injection vaccines, which pays painful procedures and may lead to spread of infectious agents via contaminated syringes (3). Nasal routes and oral routes are most accepted and easily accessible compared to the different routes of mucosal vaccination. Moreover, nasal mucosa having large number of microfold-cells (M-cells) nasal-associated lymphoid tissue (NALT), which specifically up-take the antigen-presenting cells and also induces both mucosal and systemic immune responses after immunization (4-6). Several approaches investigated to enhance the antigens uptake and improve the immunogenicity of nasal vaccines with adjuvant such as cholera toxin (7), liposomes (8) and Micro and nano-particulates (9).

Several studies have shown that micro particles based nasal formulation offers many advantages over other dosage forms (5,10). In which, biodegradable polymeric particles such as PLGA and PLA polymers have emerged as promising candidates that provides controlled release and also having their inherent immune-adjuvant property (11-12). PLGA alone having the rate limiting factor such as poor mucoadhesiveness and immune-enhancing ability and it's overcome by incorporation of mucoadhesive polymers such as chitosan to the delivery system. But chitosan has a major drawback that at physiological pH, fails to enhance permeability due to lower solubility and low positive surface charge. To tackle these problems, chitosan derivatives with improved solubility or bioadhesive properties should be employed. There are different Chitosan derivatives e.g., Thiolated chitosan, PEG-g-chitosan, Tri methyl chitosan (TMC) and Mono-N-carboxymethyl chitosan (CMC) are commonly used for nasal based formulation (13-16). In which, both TMC and CMC have been shown to exert mucoadhesive properties and excellent absorption enhancing effects even at neutral pH (17-19) compared to other chitosan derivatives. To date, several studies have used chitosan and its derivatives as coating material for HBsAg loaded microspheres, but the combinational use of TMC-CMC as a coating material has been overlooked. The specific intent of the present study was to compare the efficacy of chitosan derivative combination coated PLGA microspheres for nasal immunization. In this study, PLGA microspheres were prepared and coated using different coating agents viz., chitosan, TMC and combination of TMC-CMC. The HBsAg antigen loaded coated and uncoated microspheres were administered intranasally to mice and the immune response was determined.

2.0. MATERIALS AND METHODS

2.1. Materials:

Poly(lactic-co-glycolic acid) (PLGA) polymer, Chitosan, Trimethyl chitosan and N-carboxy methyl chitosan were purchased from Sigma aldrich. Recombinant HBsAg was kindly gifted by one of the private biotech company from Hyderabad. Aluminium hydroxide was purchased from Brenntag Biosector (Denmark). 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 Surface-modified PLGA microspheres:

Surface-modified PLGA microspheres were prepared by a modified double emulsion solvent evaporation process (20). In brief, a primary emulsion (water-in-oil) was formulated by emulsifying aqueous phase containing recombinant HBsAg, 1.5%(w/v) Trehalose, 2%(w/v) HP-β-CD and 2%(w/v) Mg(OH)2 with 4% w/v PLGA in methylene chloride using a probe sonicator (Soniweld, India) for 1 min. To this water-in-oil emulsion, 10% w/v of aqueous polyvinyl alcohol containing 0.5%w/v of Coating polymers (chitosan, TMC and combination of TMC and CMC), 0.5% Trehalose and 1% HP-β-CD 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. Chitosan was dissolved in acetate buffer (pH 4.4),

whereas TMC and CMC were dissolved in distilled water. The resultant emulsion was stirred vigorously for 3 h to evaporate the organic phase and to obtain the microspheres, which were collected by centrifugation at 22,000 g and washed twice with distilled water to remove PVA. The microspheres were then subjected to lyophilization. Uncoated PLGA microspheres were also prepared with 10% PVA solution. Alum based HBsAg was prepared as per Arash Mahboubi et al method (21) and used as control for Immunogenecity studies.

2.3. Surface Morphology by Scanning Electron Microscopy

Surface morphology of the particles was examined by using scanning electron microscopy (SEM) (LEO-435 VP, Cambridge, UK). One drop of the suspension was placed on a gold-coated plate and maintained at least 10-12 hours at room temperature in desiccators for complete dryness of the sample. Then the stub was coated with gold using sputter coater. The sample was randomly scanned and photos were taken.

2.4. Particle Size and Zeta Potential

The mean diameter and size distribution profiles of the micro particles are determined by dynamic light scattering using Malvern zetasizer Nano ZS 90 (Malvern apparatus, UK). The same instrument was used to evaluate the zeta potential of the formulations, based on electrophoretic mobility of the micro particles in diluted aqueous suspensions. Normal saline was used as the dispersion medium for the determination of particle size, whereas 1mM HEPES buffer was used for the measurement of zeta potential.

2.5. Protein Loading Efficiency

The loading efficiency/entrapment efficiency of the antigen in microspheres was determined by dissolving 20 mg of the microspheres 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 based coated and uncoated microspheres were calculated from the following equations:

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

2.6. Assessment of Structural Integrity of HBsAg

The structural integrity of HBsAg loaded microspheres was detected by SDS polyacrylamide gel electrophoresis (PAGE) and compared with the native HBsAg and reference markers. First, HBsAg was extracted from microspheres by dissolving in 2 ml of 5% (w/v) SDS in 0.1 M sodium hydroxide solution (22). 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.

2.7. Adsorption of Mucin on Microspheres

The mucin adsorption ability of uncoated and coated PLGA microspheres formulations was determined by the procedure of Filipovic-Grcic et al. [23]. Briefly, equal volumes of microspheres (2 mg/mL) and an aqueous solution of mucin (0.5 mg/mL) were mixed followed by vortexed at room temperature for 60 min. The suspension was then centrifuged, and the supernatant was quantified for free mucin content using colorimetric assay [24]. The mucin adsorbed on the microsphere surface was calculated by subtracting the amount of free mucin from the total mucin content.

2.8. *In Vitro* mucoadhesion studies

The mucoadhesive properties of HBsAg loaded microspheres were evaluated by quantity of microspheres sticking to a nasal mucosa after certain time intervals. HBsAg loaded uncoated and coated PLGA microparticle formulations were determined by the procedure of Mahajan et al. [25]. In brief, freshly cut 2cm^2 piece of sheep nasal mucosa was washed with isotonic saline solution. 100mg of HBsAg loaded microsphere were placed on mucosal surface

was fixed over polyethylene support. The microspheres were brought in contact with simulated nasal electrolytes (SNES: aqueous solution containing 8.77 mg/ml NaCl, 2.98 mg/ml KCl and 0.59 mg/ml CaCl2 per liter). The nasal mucosa was washed with phosphate buffer (pH 6.6) at the rate of 5 ml/min using a peristaltic pump. After 60 minutes application of microspheres, the concentration of antigen in collected perfusate was determined colorimetrically. The *In vitro* mucoadhesion behaviour of the microspheres was expressed as the percentage of microspheres as per following formula.

$$\% \text{ Mucoadhesion} = \frac{\text{Amount of HBsAg in washout liquid}}{\text{Actual amount of HBsAg in applied microspheres}} \text{ X } 100$$

2.9. *In Vitro* antigen release test

HBsAg drug release was estimated using *In vitro* dissolution medium. Briefly, 40 mg of microspheres 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 *In vitro* release of HBsAg. During each withdrawal, 1.0 ml of fresh PBS (pH 7.4) was again added to maintain the sink conditions.

2.10. Immunological study:

2.10.1. Measurement of specific IgG and IgA response:

Anti-HBsAg antibodies in blood samples were determined by using an enzyme-linked immunoassay (ELISA). The samples were collected from mice for immunological study (IgG and IgA) according to the method reported by Jaganathan et al., (20).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 and IgA (1:1,000 dilution, Sigma, USA) antibodies were added to well for the determination of IgG and IgA titer, respectively. The plates were kept for 1 h at room temperature and then washing was repeated. One hundred microliters of tetramethyl benzidine (TMB-H_2O_2) solution was added to each well. Color development was stopped after 30 min by adding 50 µl of 1 N H2SO4 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.

2.10.2. Estimation of cytokines levels:

Cytokine levels such as interferon-γ (IFN- γ) and interleukin-2 (IL-2) were determined according to the splenocyte proliferation assay methods (26, 27) with slight modifications. In brief, the rat spleens were weighed and homogenized with CHAPS 1% (Sigma. USA) in ice-cold PBS and 10% (w/v) homogenates were obtained with the help of tissue homogenizer. Homogenates were incubated for two hours in an ice-bath then the supernatant was centrifuged at 2000 X g for twenty minutes. The supernatant solution was used for the measurement of Cytokine levels (IL-2 and IFN- γ) by ELISA method.

2.11 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 GraphPad InStatTM software (GraphPad Software Inc., San Diego, California). A value of $P < 0.05$ was considered to be statistically significant.

3.0. RESULTS AND DISCUSSION:

3.1. Particle size, surface morphology and zeta potential

Coated and un-coated HBsAg microspheres were prepared using the modified double emulsion method. The microspheres uptake by nasal epithelial and NALT cells depends on particle surface charge and size (28, 29). Uncoated microspheres showed negative zeta potential whereas a coated microsphere (chitosan, TMC and combination of TMC-CMC) has shown positive zeta potential. A TMC and TMC-CMC microspheres exhibited slighter increase in zeta potential as compared to chitosan microspheres. The surface morphology of the coated microspheres was studied by SEM. The study revealed that most of the microspheres were approximately spherical in shape having a smooth surface (Fig-1). The particle characteristics of uncoated, chitosan coated, TMC coated and TMC-CMC microspheres were shown in Table I. The antigen loading efficiency was also comparable with both coated and uncoated microspheres (Table I).

Table 1: Particle size, zeta potential and % loading efficiency of HBsAg loaded Coated and uncoated PLGA microspheres

Microparticle	Particle size (µm)	Poly dispersity index	Zeta potential (mV)	% Loading efficiency
Uncoated	4.64 ± 0.03	0.072 ± 0.002	-11.1 ± 0.3	82 ± 3
Chitosan coated	5.13 ± 0.06	0.112 ± 0.005	18.1 ± 2.7	79 ± 5
TMC coated	5.32 ± 0.11	0.128 ± 0.007	27.4 ± 3.2	82 ± 4
TMC-CMC coated	5.67 ± 0.15	0.132 ± 0.009	26.8 ± 1.8	81 ± 3

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

3.2. Confirmation of the Structural Integrity of the Antigen

During the microspheres 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 microspheres, we used trehalose and HP-β-CD as stabilizer and $Mg(OH)_2$ as acid neutralizing agent. Structural integrity of the entrapped antigen in microspheres was assessed using SDS-PAGE (Fig.2). The SDS-PAGE analysis revealed that the native antigen and antigen released from the uncoated and coated formulation demonstrated identical bands. This confirmed that structural integrity of antigen remains stable during the encapsulation and release process.

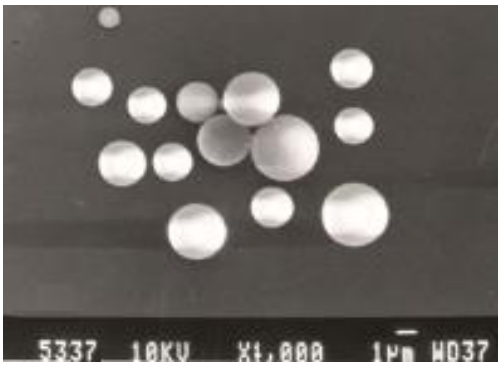

Fig 1: SEM Picture of TMC-CMC coated microspheres

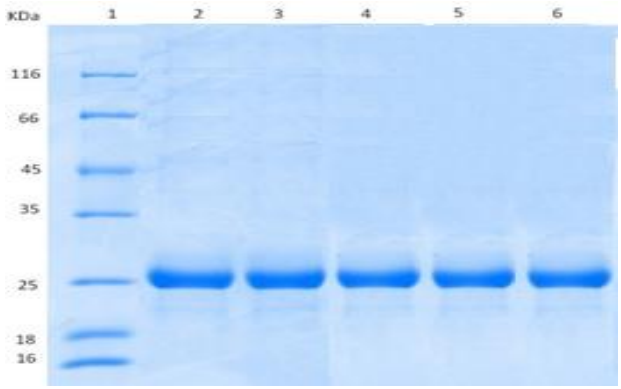

Fig 2: SDS–PAGE analysis: SDS–PAGE showing stability of antigen isolated from formulations. (Lane 1) Marker proteins, (Lane 2) native HBsAg (24 kDa); (Lane 3–6) HBsAg isolated from uncoated PLGA, Chitosan-PLGA, TMC-PLGA and TMC-CMC-PLGA microspheres respectively (24 kDa).

3.3. Adsorption of Mucin on microspheres

Coated and uncoated PLGA microspheres were evaluated for their mucoadhesiveness of the body surface. Earlier investigators report that positive surface charge may be favorable in nasal vaccination due to their better mucoadhesive ability (30). Mucin adsorption (milligram/milligram) of particle was 0.011±0.002, 0.132±0.012, 0.246±0.023 and 0.268±0.014 for uncoated, chitosan coated, TMC coated and TMC-CMC coated microspheres, respectively. Microspheres with Chitosan, TMC and TMC-CMC show better mucin retention ability as compared to that of uncoated microspheres. And it was observed that substantially that high mucin adsorption with TMC and TMC-CMC as compared to chitosan coated microspheres. Therefore, it can be assumed that residence time of uncoated microspheres in the nasal cavity is low due to lack of mucoadhesiveness. In case of coated microspheres, better immune-adjuvant effect observed for TMC-CMC and TMC over chitosan may be due to the high positive charge.

3.4. *In vitro* mucoadhesion of HBsAg loaded microspheres

The mucoadhesive percentage of coated and uncoated microspheres to nasal mucosa was evaluated using the sheep nasal mucosa and the results are shown in Table 2. Microspheres with coated i.e. Chitosan, TMC and TMC-CMC show better mucoadhesive as compared to that of uncoated microspheres. Mucoadhesion may occur due to interaction forces, such as electrostatic attraction and hydrogen bonding. In the case of chitosan and its derivatives based microspheres may acts through the electrostatic attraction because of the negatively charged sialic acid of the mucin and the positively charged glucosamine residues of chitosan (31). It was observed that TMC-CMC showed the highest range of mucoadhesive percentage as compared to that of Chitosan and TMC. It could be due to the good bio-adhesive property of CMC.

Table 2: *In-vitro* mucoadhesive tests: percentage of microspheres attached

Microparticle	Percentage of *in vitro* mucoadhesion
Uncoated	45 ± 4.2
Chitosan coated	78 ± 2.6
TMC coated	82 ± 3.2
TMC-CMC coated	88 ± 2.2

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

3.5. *In Vitro* antigen Release profile

In microparticle formulations, stabilizers and neutralizer such as trehalose, Hydroxy propyl-β- cyclodextrin and magnesium hydroxide 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 uncoated and coated microspheres was determined in PBS (pH 7.4). Both coated and uncoated microspheres formulation shows an initial burst release followed by a prolonged release shown in figure 3. No major difference was observed in antigen release profile shows of both coated and uncoated microspheres.

3.6. Immunological study:

The immune effect of the developed formulations investigated using the different groups of animals were immunized with uncoated, chitosan coated, TMC coated, TMC-CMC coated microspheres (intranasally) and alum adjuvanted HBsAg (subcutaneous). Serum IgG and secretory IgA were determined using ELISA. It was detected that intranasal immunization of HBsAg associated with coated microspheres shows high antibody titer as compared to the uncoated HBsAg ($p < 0.05$) shown in Fig 4. Results showed that TMC-CMC shows significantly higher IgG titer as compared to Chitosan coated microspheres ($p < 0.05$). So, the better local and systemic uptake of the TMC-CMC and TMC coated microspheres, which resulted into the stronger and persistent immune response following nasal administration. And also observed, strongest serum IgG titer produced after vaccination of alum based HBsAg.

Mucosa is the largest area of the body exposed to the external pathogens such as virus, bacteria, fungi and parasites. These pathogens are protected against the mucosally applied antibodies and also proven that mucosally applied antibodies elicit cross protective immunity more effectively than serum IgG (32). Specific sIgA was determined in local (nasal) and distal (vaginal and salivary) secretions shown in Fig 5. The results of sIgA responses level were significantly higher in case of TMC-CMC and TMC coated microparticle as compared to uncoated and Chitosan coated microspheres ($p < 0.05$). It may be due to the better solubility and penetration-enhancing ability at physiological pH of TMC-CMC and TMC microspheres. And also observed that alum based HBsAg could not induce significant serum IgG titer in local and distal secretions. Significant endogenous cytokine levels (IL-2 and IFN-γ) were observed in mice immunized with coated PLGA-based formulations as compared to those control and uncoated PLGA-based formulations shown in Fig 6 and 7 ($p < 0.05$). Mucosal, humoral and cellular immunity were recorded significantly stronger with TMC-CMC coated modified PLGA microspheres when compared with other coated and uncoated PLGA microspheres administered intranasally ($p < 0.05$).

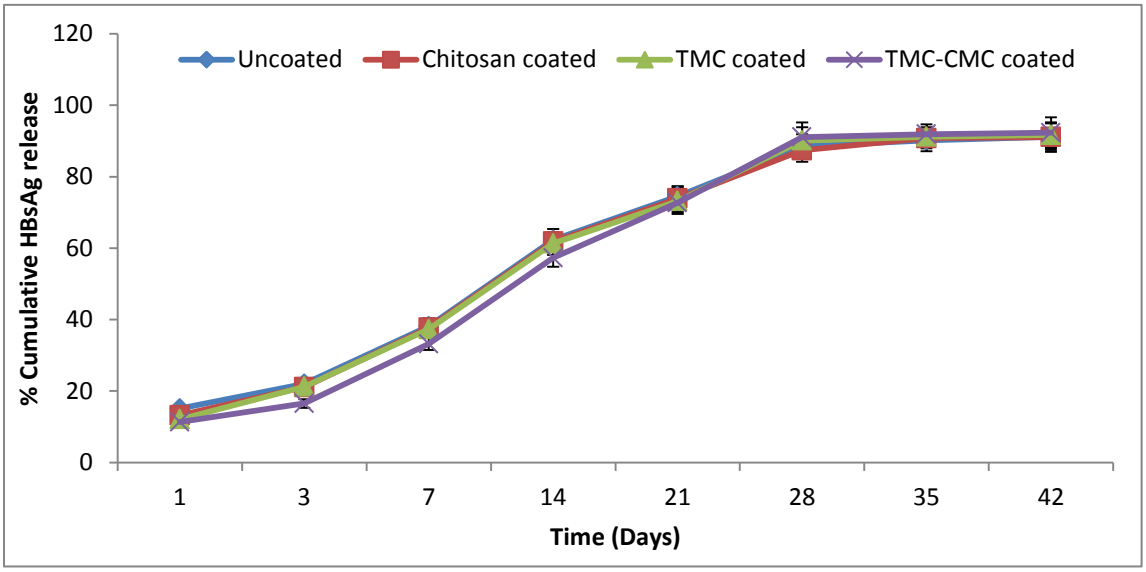

Fig 3: *In vitro* release study of % antigen release with respect to time in PBS (pH 7.4). Values are expressed as mean ± S.D. (n = 6).

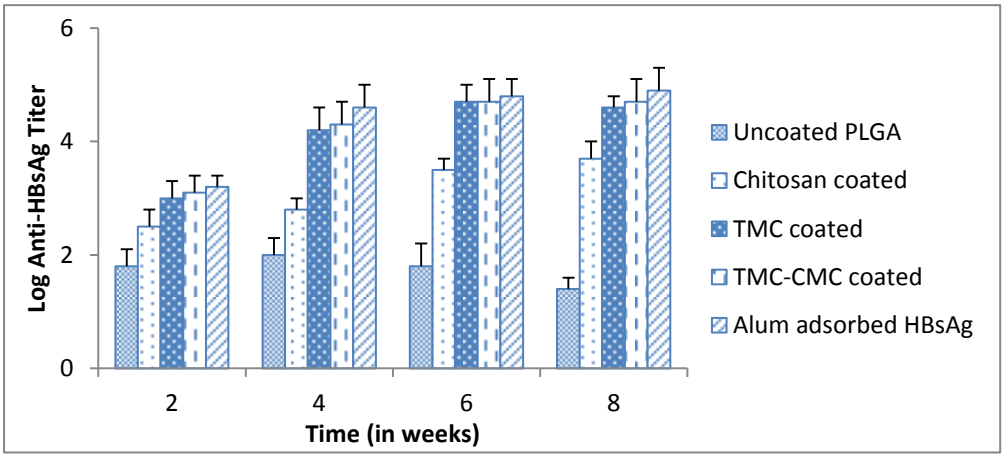

Fig 4: Anti-hepatitis B surface antigen IgG titer in serum. Values are expressed as mean ± S.D. (n = 6).

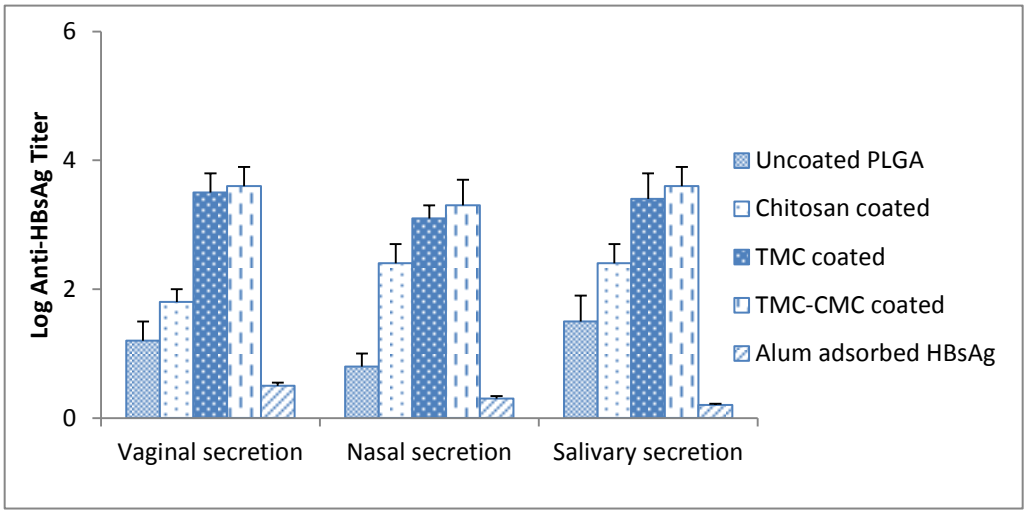

Fig 5: Anti-hepatitis B surface antigen sIgA titer in nasal, salivary, and vaginal secretions. Values are expressed as mean ± S.D. (n = 6).

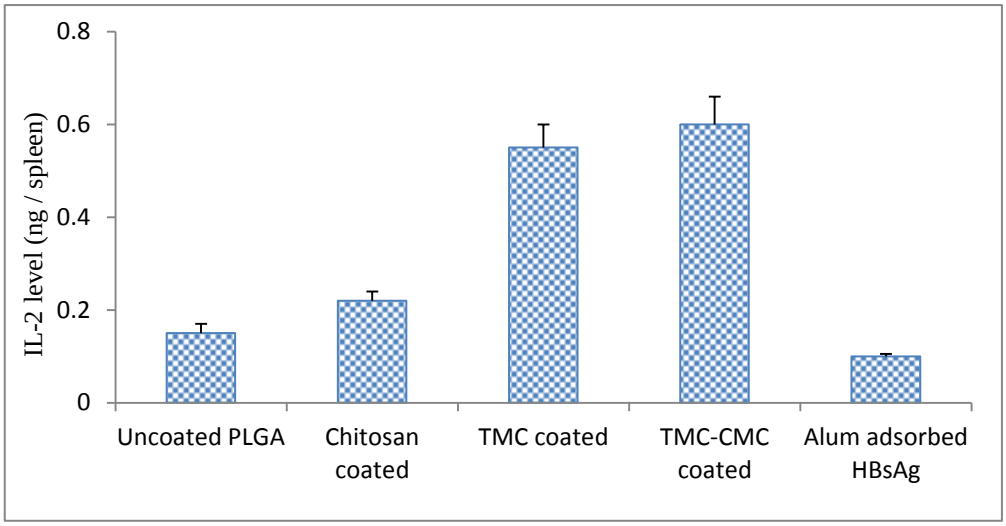

Fig 6: Interleukin-2 level in the spleen of mice immunized with PLGA-based formulations. Values are expressed as mean ± S.D. (n = 6).

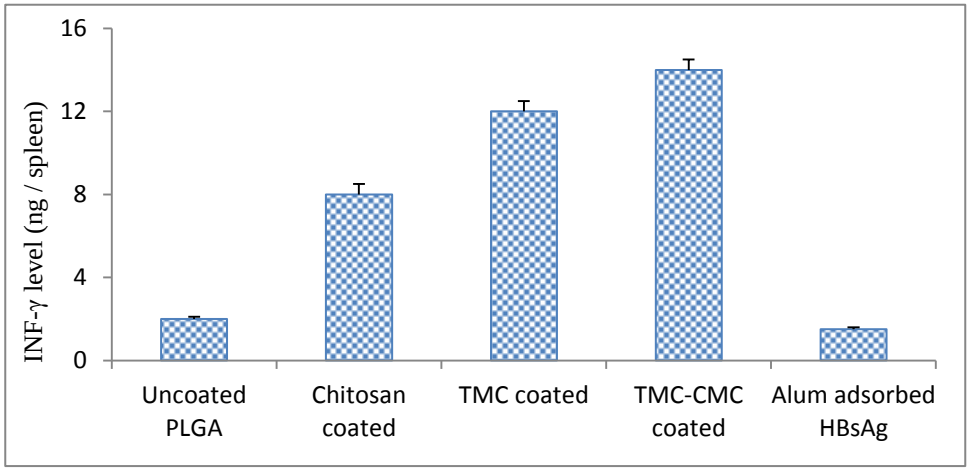

Fig 7: Interferon-γ level in the spleen of mice immunized with PLGA-based formulations. Values are expressed as mean ± S.D. (n = 6).

4.0. CONCLUSION:

Mucoadhesive property of uncoated and coated PLGA microspheres were explored in this study and the results evidenced that the better immunogenicity was produced with coated PLGA microspheres of HBsAg after intranasal immunization. We observed coated microspheres made of chitosan derivatives (TMC and TMC-CMC) induced strong immune-adjuvant effect compared to others, due to the positive charged surface area and better solubility. And also observed slightly superior behavior observed with TMC-CMC compared to TMC microspheres, it may be due to good mucoadhesive effect and enhanced penetration ability at physiological pH from the polymer combination. Finally on the basis of *In vitro* and *In vivo* studies, we can conclude that HBsAg loaded TCM-CMC microspheres is a good alternative carrier for mucosal vaccine delivery.

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