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Viral protein complexed liposomes for intranasal delivery of hepatitis B surface antigen

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ABSTRACT

The present work investigates prospective of recombinantly expressed influenza surface protein haemagglutinin (HA) complexed liposomes for intranasal delivery of HBsAg. Liposomes encapsulating HBsAg were prepared and complexed with HA. The prepared formulations were extensively characterized for vesicle size, polydispersity index, entrapment efficiency, HA complexation efficiency, *in vitro* release, etc. Stability of protein molecules was accessed by SDS-PAGE. The antigenicity of protein HBsAg was determined by EIA and the functional stability of HA was evaluated by haemagglutination assay. Subsequently, *in vivo* study was carried out to study their feasibility as nasal vaccine carriers. A significant and perdurable immune response was obtained following *in vivo* administration of the developed formulations that was comparable with alum adsorbed HBsAg administered intramuscularly. The HA complexed liposomal formulations elicited sIgA in mucosal secretions and also demonstrated cellular immune response both of which are not induced in the case of alum adsorbed HBsAg vaccine. Further, the HA complexed liposomes produced higher immune response as compared to plain liposomes that might be due to higher uptake of former as evidenced in microscopy study of nasal tissues. The higher cellular response generated by HA complexed liposomes may be possibly due to characteristic pH dependent fusion property of HA protein.

1. Introduction

Mucosal vaccination is an attractive alternative of parenteral vaccination as it offers a number of advantages. It augments immunity in mucosal compartments which is the prime portal of entry for the majority of pathogen (including HIV, hepatitis and influenza) and the immunity generated in this way is also transferred to systemic compartment. In contrast the immunity generated *via* parenteral immunization cannot be transferred to mucosal surfaces. Further, being needle free way of immunization mucosal route suits for mass vaccination especially in developing countries where transmission of infection owing to the reuse of needle has been a problem (Nossal, 2000). Success of the oral Sabine polio vaccine established the efficiency of mucosal route and subsequently a number of efforts have been made to develop mucosal vaccine against other pathogens. These include live vector based vaccines and recombinant protein antigen or DNA based vaccines. Although attenuated pathogens and live vectors based mucosal vaccines are highly effective, there are safety concerns such as reversion to virulence, raising of innate and severe inflammatory immune responses by live pathogens and in case of intranasal (i.n.) administration, transport of the pathogen/vector to the brain (Holmgren et al., 2003). Vaccine based on killed, whole pathogen cell are somewhat effective but these can potentially contain toxic molecules such as lipopolysaccharides or live pathogens (Ryan et al., 2001).

Considering these issues a significant research focuses on developing mucosal vaccine based on subunit antigen developed by rDNA technology. Nevertheless, these antigens are poorly immunogenic (Neutra and Kozlowski, 2006; Ogra et al., 2001) and therefore require mucosal adjuvant or carrier delivery system to be effective. These include mainly cholera toxin (CT), *Escherichia coil* heat labile toxin (LT), CpGODN, DNA, virosomes, liposomes, chochelates, polymeric microspheres, mucoadhesive polymers, immunostimulating complexes and virus like particles (Holmgren et al., 2003; Lemoine et al., 1998; Ryan et al., 2001; Kemble and Greenberg, 2003). Among the available options, liposomes has gained most significant attention as a mucosal vaccine delivery system. These carriers offer several advantages including protection of antigen during transit through mucosal surfaces and facilitate antigen uptake by M cells (Aramaki et al., 1994). Furthermore, they are easy to prepare, characterize and also stabilize the antigen following encapsulation. Beside being biocompatible and biodegradable, the liposomes have

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proven to augment effective and durable immune response against the associated antigen (Khatri et al., 2008).

In the context for delivery of mucosal vaccines, nasal and oral delivery route are most extensively studied. The oral route although being most acceptable from the view of patient compliance is associated with low pH of stomach, higher proteolytic activity at the GIT, requirement of large dose and these factors renders development of oral vaccine difficult. On the other hand nasal route is more advantageous as it is devoid of the above drawbacks. Also a lower dose of antigen or adjuvant is required probably due to relatively higher antigen uptake and low degradation (Holmgren et al., 2003; Lemoine et al., 1998; Yuki and Kiyono, 2003). However, a careful selection of adjuvant is required owing to the presence of olfactory nerve and any accumulation of toxic adjuvant may lead to central nervous system toxicity and pathogenic consequences such as facial palsy (Mutsch et al., 2004). The carrier system such as liposomes primarily convert a soluble antigen into particulate one and these particulate antigen basically the mimic of pathogens which are efficiently taken up by the cells of immune system.

In the case of mucosal vaccine development, M cells are the primary portal of antigen sampling (Tyrer et al., 2006). These cells exploit receptor dependent transport for bacterial and viral pathogen. In addition dendritic cells also extend their dendrites between the cells of intestinal epithelium and capture antigen for presentation to the lymphocytes (Rescigno et al., 2001). Further, the uptake of particulate material is dependent on its size (Fujii et al., 1993) as well as surface chemistry. The most attractive motif of the approach is pathogen mimicking molecular designing of the carrier systems so as to make it similar to the pathogenic organism. Any of the carrier particles decorated with a molecule from pathogenic origin (or a protein present on the surface of bacteria or virus) which mediates its interaction and internalization by mucosal surfaces is expected to enhance the interaction of carrier with mucosal surface.

In the present investigation we have selected the recombinantly expressed haemagglutinin protein that is present on the surface of influenza virus. This protein mediates interactions and transmucosal transport of virus with mucosal surface (Wenxin Wu and Gillian, 2004; Lakadamyali et al., 2004). This may be attributed to its binding affinity to sialic acid receptor present on mucosal surface (Stevens et al., 2006) and cytosolic release of viral components which is mediated by the fusion between vial and endosomal membrane thereby releasing the content in cytosol (Sammalkorpi and Lazaridis, 2007; Lakadamyali et al., 2004; Lee et al., 2006). Further, the studies revealed that the M cells play a significant role in internalization of the influenza virus as abundant receptors for influenza virus interaction are present on these cells (Fujimura et al., 2004; Kumlin et al., 2008).

The present study very first time investigates the prospective of influenza protein HA complexed liposomal constructs for intranasal delivery of model antigen HBsAg (hepatitis B surface antigen). The HA complexed, HBsAg loaded liposomes were prepared, characterized and optimized for various *in vitro* parameters. The structural stability of antigen HBsAg was measured by SDS-PAGE analysis and enzyme immunoassay. The stability and functional reactivity of HA protein was measured by SDS-PAGE and haemagglutination assay. *In vivo* immunogenicity of the vaccine following intranasal administration was evaluated by measuring systemic (IgG and cytokine response) as well secretory (IgA) immune responses.

2. Materials and methods

The protein supplied as Baculovirus-derived recombinant influenza haemagglutinin (rHA; H1N1 strain A/Texas/36/91) was obtained from Protein Sciences Corporation (Meriden, CT). Distearoylphosphotidyl choline (DSPC), cholesterol (CH) and 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate hydrate (CHAPS) were purchased from Sigma Chemical Co. (St Louis, MO, USA). 1-(3-Dimethylaminopropyl)-3-ethyl carbodiimide (EDC) and N-glutarylphosphatidyl ethanolamines (NGPE) were procured from Fluka Chemica Co. (AG CH-9470 Buchs, Switzerland) and Avanti Polar Lipid, Inc., respectively. HBsAg (MW 24 kDa, 1.5 mg/ml) was obtained from Shantha Biotech Ltd. (Hyderabad, India). Enzyme linked immunoassay kit (Ausab and Auszyme) was purchased from Abbott Laboratories, USA and cytokines estimation kit was obtained from e-Bioscience. The FITC conjugation kit and BCA protein estimation kit was purchased from Genei (Banglore, India). All other chemicals and reagents were of analytical grade.

2.1. Preparation of liposomes

Liposomes were prepared with distearoylphosphatidyl choline (DSPC), cholesterol (CH) and NGPE by reverse phase evaporation method as described by Sazoka and Papahadjopoulos (1978) with slight modifications. Briefly lipids, were dissolved in 5 ml diethyl ether to which 2 ml of aqueous phase, i.e., phosphate buffer saline containing antigen was added and sonicated (Soniweld, India) for 2 min at 4 °C. The resultant mixture was placed into rotary evaporator (Steroglass, Italy) until thick gel was formed. Subsequently, the dispersion was kept over a vortex mixer under nitrogen environment in order to remove any residual ether. The obtained suspension was pushed through polycarbonate membrane filter of 0.8 μm (Nucleopore, The Netherlands) to control size of the vesicles. Finally, the unentrapped antigen from the prepared liposomes suspension was removed by using sephadex minicolumn.

2.2. Preparation of HA complexed liposomes

The recombinant protein haemagglutinin (HA) from H1N1 strain of influenza virus was complexed onto the surface of liposomes by the method described by Bogdanov et al. (1988) and Khatri et al. (2005) with minor modifications. Briefly, 50 μl EDC (0.25 M in water) and 50 μl of sulfo-NHS were added to 500 μl of liposomal dispersion and the mixture was incubated for 60 min. Subsequently, the HA protein solution (in PBS buffer pH 7.4) was added and resultant mixture (pH 7.4) was incubated for 3 h with mixing. The unbound protein was removed by gel filtration using sepahadex-100 mini column (Hermanson, 1996).

2.3. Characterization of liposomes

The liposomal formulations were characterized for their shape and morphology by transmission electron microscopy (JEM-200 CX, JEOL, Tokyo, Japan). Samples for microscopy were prepared by conventional negative staining method using 0.2% phosphotungstic acid. Specimens were prepared by dropping the dispersion onto carbon-coated EM grids. The grid was held horizontally to allow the molecular aggregates to settle and then tilted to 45° for a while to drain the excess fluid. Afterward, drop of phosphotungstic acid (pH 4) was added to the grid to give a negative stain. The grid was then kept aside for 20 s before removing excess stain as above. Specimens were air-dried before and examined using transmission electron microscopy (Philips Morgagni, Netherlands). Particle size of liposomal suspension was measured by photon correlation spectroscopy (Nano ZS 90, Malvern, UK) at 25 °C by diluting the liposomal dispersion to the appropriate volume with PBS buffer pH 7.4. Zeta potential (an indirect measurement of surface charge) of various formulations was measured using same instrument.

2.4. Entrapment efficiency

Prepared liposomes were separated from the free (unentrapped) antigen by Sephadex G-100 minicolumn using centrifugation technique (Fry et al., 1978). The method was repeated thrice subsequently minimum amount of Triton X-100 (0.1%, w/v) was added to disrupt the vesicles. The liberated antigen was estimated by BCA (bicinchoninic acid) protein assay and the percent vesicular friction of antigen entrapment was determined. The protein content of HA complexed liposomes was determined with the method reported by Tardi et al. (1997). Briefly, the protein content of HA anchored liposomes (a), HBsAg encapsulated plain liposomes (b) and HA coupled placebo liposomes (without entrapped antigen HBsAg) (c) was determined by micro BCA protein assay. The difference of two value (i.e., a and b) represent the amount of HA complexed to liposomes whereas the difference between a and c represents the amount of HBsAg loaded in HA complexed liposomes. In addition, the HBsAg content of various liposomal formulations was also determined by enzyme immunoassay (EIA) to confirm the amount of HBsAg loaded in various formulations.

2.5. In vitro antigen release

The *in vitro* antigen release profile of entrapped antigen from different liposomal formulations was studied using dialysis bag method as described by Tiwari et al. (2009). Accurately measured amount of plain or HA complexed liposomal formulations were taken into a dialysis bag (Sigma, USA of 100 kDa MWCO) and placed in a receiving compartment containing 50 ml of PBS; pH 7.4. The whole set was placed on a magnetic stirrer at $25 \pm 1\,^{\circ}C$. Samples were withdrawn at predetermined time intervals and replaced with the same volume of PBS. The withdrawn samples were assayed for antigen content by BCA protein assay method (Banglore genei Pvt Ltd., India).

2.6. In process stability study

The structural integrity of antigen extracted from liposomes was detected by SDS-Poly acrylamide gel electrophoresis (SDS-PAGE) and compared with the native HBsAg, HA and reference molecular weight markers (Tiwari et al., 2009; Singh et al., 1997). Briefly, the extracted antigen solution from plain liposomes, HA complexed liposomes, native protein and molecular weight reference markers were loaded onto a 3.5% stacking gel and then subjected to electrophoresis on 12% separation gel at 200 V (Bio-Rad, USA) until the dye band reached the bottom. Following migration the gel was stained with Coomassie blue to reveal the protein antigen bands.

To confirm the three dimensional integrity of antigen HBsAg as well as *in vitro* antigenicity of antigen EIA (enzyme immunoassay) was performed using EIA kit (AUSZYME Monoclonal kit, Abbott Laboratories). The plain antigen solution and the antigen from liposomal dispersion were analyzed for antigen content as described by Shi et al. (2002). The ratio of EIA response to protein concentration (measured by BCA method) was recorded as indicative of antigenicity.

2.7. Haemagglutination assay

Several bacterial and viral proteins including haemagglutinin are able to agglutinate human or animal RBC and bind to N-acetyl neuraminic acid and RBCs form a lattice that may be directly observed visually (Wu et al., 2009; Killian, 2008). In the present investigation haemagglutination assay was performed to evaluate the agglutination ability of HA protein. The serial dilution of liposomes coupled HA protein (100 μl) and the equal concentration of plain HA protein in PBS buffer were incubated with equal volume in wells of V bottom PVC plates (Tarsons, India). Chicken red blood cells were added to the wells and incubation was continued on a gentle rocking plate for 30 min at room temperature. Lattice formation was scored visually as an evidence of haemagglutination (Singh et al., 2004).

Table 1
Immunization protocol to evaluate the immune response induced following administration of various formulations in BALB/c mice ($n = 6$).

Formulation	Formulation code	Rout of administration
HBsAg in PBS (pH 7.4)	Pl-HBsAg	IN
Alum adsorbed HBsAg	Alum-HBsAg	IM
Liposomes encapsulated HBsAg	Lipo(HBsAg)	IN
HA complexed liposomes encapsulated HBsAg	Lipo(HBsAg)-HA	IN

IM = intramuscular, IN = intranasal.

2.8. Fluorescence microscopy studies

To investigate the interaction of prepared liposomal formulations with nasal mucosa fluorescent microscopy study was performed as per the guidelines of institutional animal ethical committee. Briefly, FITC labeled HBsAg (FITC-HBsAg) encapsulated liposomes were prepared by the same procedure as described elsewhere in text for preparing antigen loaded plain and HA complexed liposomes. The labeling of HBsAg with FITC was done using FITC labeling kit (Genei, Banglore, India) according to manufacturer instructions. The liposomal formulations were administered intranasally (2 μl in each nostril) to fully awake mice. The control group received equivalent dose of FITC-HBsAg dissolved in PBS buffer (pH 7.4). After 2 h of administration of various formulations the mice were sacrificed by cervical dislocation and nasal mucosa were excised, fixed and processed for microtomy. Sections of about 3 μm thickness were then observed under fluorescent microscope (Nikon, Japan).

2.9. In vivo immunization study

In vivo immunization study was conducted to evaluate the immunization potential of developed vaccine formulation. The study was carried out as per the guidelines compiled by CPCSEA (Committee for the Purpose of Control and Supervision of Experiments on Animal, Ministry of Culture, Government of India). The animals were kept under standardized conditions at the Animal Care facility of Department of Pharmaceutical Sciences, Dr. H.S. Gour University, Sagar, (MP), India, with food and water *ad libitum*. BALB/c mice were used for immunization study with each group comprising of 6 animals. To evoke an immune response 4 μl (2 μl/nostril) of antigen in PBS and antigen loaded various liposomal formulations consisting of 10 μg antigen/dose (immunization protocol is given in Table 1) was inoculated intranasally in small drops. Dosing was performed using sterile micropipette tip into the nostril of the non-anesthetized animal (supine position) and ejecting the formulation into the nasal cavity. Care was taken that subsequent drop was only given when the former had been entirely inspired. Alum adsorbed formulation of antigen was also administered intramuscularly contain 10 μg antigen/dose. Further, the dose of HA in the case of Lipo(HBsAg)-HA was fixed to 5 μg of the antigen/dose.

2.9.1. Sample collection

Following immunization samples were collected from various body fluids by following standard protocol. For collection of serum sample blood was collected by retro-orbital puncture (under mild ether anesthesia) after 2, 4, 6 and 8 weeks of primary immunization and sera was stored at −40 °C until tested by ELISA for antibody. The nasal, vaginal, intestinal and salivary secretions were collected after 6 weeks of primary immunization. A vaginal wash was obtained according to the method reported by Debin et al. (2002). Briefly, 50 μl of PBS containing 1% (w/v) bovine serum albumin (1% BSA in PBS) was introduced into the vaginal tract of non-anesthetized mice using a Gilson pipette. These 50 μl aliquots were withdrawn and reintroduced nine times. The nasal wash was similarly collected by cannulation of the trachea of sacrificed mice. The nasal cavity was then flushed three times with 0.5 ml of 1% BSA in PBS (pH 7.4). Salivation was induced as described in our previous studies (Khatri et al., 2008; Mishra et al., 2010). Mice were injected with 0.2 ml sterile pilocarpine solution (10 mg/ml) intraperitoneally. The saliva from mice was collected after 20 min using capillary tube. Intestinal lavage was performed using the technique reported by Elson et al. (1984). Briefly, four doses of 0.5 ml lavage solution (NaCl-25 mM, Na_2SO_4-40 mM, KCl-10 mM, $NaHCO_3$-20 mM and polyethylene glycol Mw 350; 48.5 mM) were administered intragastrically at 15 min intervals using a blunt tipped feeding needle. Thirty minutes after the last dose of lavage solution, the mice were given 0.2 ml pilocarpine (10 mg/ml) intraperitonially. A discharge of intestinal contents occurred regularly over next 20 min, which was collected carefully. These fluids were stored with 100 mM phenylmethyl sulfonyl fluoride (PMSF) as a protease inhibitor at −40 °C until tested by ELISA for secretory antibody (sIgA) levels.

2.9.2. Determination of antibody titer by ELISA

Specific antibodies (IgG) against antigen (HBsAg and HA) were measured by ELISA. ELISA plates (Nunc-Immune plat Fb96 Maxisorb, India) were coated with antigen (100 ng per well) stored overnight at 4 °C and then blocked with buffer containing 5% skim milk in 1% tris buffer saline for 2 h at room temperature. Diluted sera were added in triplicate to antigen coated wells and incubated for 2 h at room temperature. After washing with 0.1% tween-20 in tris buffer saline (PBST), plate were incubated with horseradish peroxidase (HRP) conjugated secondary anti-mouse antibody for 2 h at room temperature. Plate was again washed with PBST and then 100 μl of substrate solution 3,3′,5,5′-tetra methyl benzidine containing hydrogen peroxide was added to each well. Plate was incubated in darkness at room temperature for 15 min. The reaction was stopped by adding 50 μl of 2 M H_2SO_4 to each well. Absorbance was measured at 405 nm using micro plate ELISA reader (Bio Rad). The end point titer was expressed as logarithm of the reciprocal of the last dilution, which gave an optical density at 405 nm above the optical density of negative control. Similarly the concentration of IgA was determined in seretory fluid using HRP conjugated goat anti-mouse IgA (Sigma, USA). Antigen-specific IgG isotype titers in mice were determined by ELISA assay using antibody conjugates to specific IgG subclasses. The HRP conjugates used were goat anti-mouse IgG1, IgG2a (Sigma, St. Louis, MO, USA).

2.9.3. Estimation of cytokine level

In vitro cytokines level in the immunized animals was determined by ELISA as described previously (Nakane et al., 1992). Briefly the spleens were aseptically removed from euthanised animals and a spleen cells suspension was prepared as mentioned elsewhere (Perrie and Gregoriadis, 2000). These cell homogenates (100 ml) obtained from the four mice of each group were plated individually in 96-well tissue culture plates (Fisher, UK) in triplicate, along with 100 ml of RPMI 1640 (Himedia) containing soluble HBsAg at the concentration of 2.5 mg/ml at 37 °C in a humidified incubator at 5% CO_2 environment. ELISA development kit (eBioscience) was used to quantify, according to the manufacturer's instructions. After 48 h of splenocyte stimulation with the soluble antigen, the cells were homogenized, centrifuged and the interleukin concentrations (IL-2, IL-4, IL-6 and interferon IFN-γ) in the supernatant.

2.10. Statistical analysis

Statistical analysis was performed on the data obtained in the *in vitro* and *in vivo* studies by one-way analysis of variance (ANOVA) with Tukey–Kramer multiple comparisons post test using GraphPad InStatTM software (GraphPad Software Inc., San Diego, CA). Throughout, the level of significance was chosen as less than 0.05 (i.e., $p < 0.05$). The post hoc test was performed only if findings of the ANOVA were significant.

3. Result and discussion

3.1. Preparation and characterization of liposomal vaccine formulations

In the present investigation viral protein complexed liposomes were used as a carrier for recombinant antigen vaccine. The liposomes were prepared by reverse phase evaporation method which is associated with high entrapment efficiency and then surface complexed with haemagglutinin protein antigen. Liposomes were prepared with various molar ratios of distearoyl phosphatidyl choline (DSPC), cholesterol (CH) and NGPE. The prepared formulations were optimized on the basis of size, polydispersity index, turbidity, zeta potential, entrapment efficiency and *in vitro* release (data not shown). The optimum concentration of lipids was found to distearoylphosphatidyl choline (DSPC), cholesterol (CH) and NGPE was 6:3:1 molar ratio. The lipid NGPE was added to the formulation for conjugation. The liposomal formulation was subsequently conjugated with HA protein to enhance uptake by nasal mucosal tissue. The liposomes were prepared by reverse phase evaporation method since it is a simple and convenient method to prepare liposomes and associated with good entrapment efficiency (Sazoka and Papahadjopoulos, 1978). Further instead of phosphotidylcholine as a main constituent for liposomes preparation, DSPC was taken owing to its higher high gel-liquid crystalline transition temperature (T_c) which accounts for its higher stability, better presentation of antigen and the higher immunization potential (Gregoriadis et al., 1992; Aramaki et al., 1994; Alpar et al., 1992).

The complexation of HA on to the surface of liposomes was done by carbodiimide coupling method. Carbodiimide involves the activation of carboxylic acid group to give NH-activated carboxylic group of NGPE which reacts with free amino group of the ligand protein HA molecule (Olde-Damink et al., 1996). The method of carbidimide coupling is fairly good method to facilitate conjugation of protein on the surface of liposomes and has been used many times. The strategy has been reported to be mild enough to maintain three dimensional structural integrity as well as functional integrity/activity of the antigenic protein molecules [e.g., gp41 epitope of HIV (Singh and Bisen, 2006) HBsAg (Khatri et al., 2005) and others (Munoza et al., 2004; Munoz et al., 1998)].

A very low polydispersity index of less than 0.2 was recorded for all the formulations indicate narrow size distribution of vesicles (Table 2). The encapsulation efficiency was expressed as percentage of antigen which was added initially for loading of vesicles. The average vesicle size and entrapment efficiency of plain liposomes was found to be 643 ± 10 nm and 53.3 ± 3.2%. Complexation of HA protein resulted in marginal increase in size of the liposomes (712 ± 12 nm) that might possibly due to surface immobilization of antigen but the entrapment efficiency was largely unaffected. Sur-

Table 2
Characterization of various liposomal formulations.

Formulation	Average vesicle size	Entrapment efficiency	HA conjugation efficiency	Zeta potential (mV)	PDI
Lipo-HBsAg	643 ± 10 nm	53.2 ± 1.2%	–	−31.17 ± 0.72	0.147 ± 0.019
Lipo-HBsAg-HA	712 ± 12 nm	51.2 ± 0.8%	45.6 ± 6.9%	−42.32 ± 0.91	0.159 ± 0.027

Mean ± SD, $n = 4$.

face morphological studies were performed in order to study the shape of prepared systems using transmission electron microscopy indicated that both HA complexed liposomes and plain liposomes were almost spherical and surface was smooth. However, no significant difference in surface morphology of HA complexed and plain liposomes was observed (Fig. 1). The zeta potential (an indirect measurement of surface charge) is dependent upon characteristics lipid used in the formulation of liposomes. In the present investigation lipids disteryphosphatidyl choline (DSPC), cholesterol (CH) and NGPE were used in the ratio of 6:3:1 for the preparation of plain liposomes and shows zeta potential of the value of −31.17 ± 0.72 mV. This may be attributed to presence of anionic NGPE in the liposomal formulation, thereby imparting negative charge to liposomal surface. The results were in accordance to earlier reports (Anabousi et al., 2005). Zeta potential of liposomes was found to be slightly higher for HA complexed liposomes (Table 2) due to presence of HA molecule on the surface of liposomes. The protein HA was negatively charged (p*I*-5.5–5.8) under experimental condition therefore the HA complexed liposomes showed slightly higher (more negative) zeta potential as compared to plain liposomes. The coupling efficiency of the protein was estimated in the supernatant of the conjugation reaction mixture as well as by preparing the HA coupled placebo liposomes analyzed by BCA protein assay method and found to be 45.6 ± 6.9% (Table 2).

The *in vitro* release profile of entrapped antigen from plain and HA complexed liposomal formulation was studied using dialysis bag method. In case of plain liposomal formulation antigen release was found to be 90.23 ± 2.10% at the end of 144 h where as in the case of HA complexed liposomal formulations the release was found to be 83.12 ± 1.72% (Fig. 2). The result of *in vitro* release study shows that the presence of protein HA on to the surface of liposomes slows the release rate of encapsulated bioactive which possibly occurs due to the presence of additional barrier provided by protein on to the surface of liposomes (Rai et al., 2008).

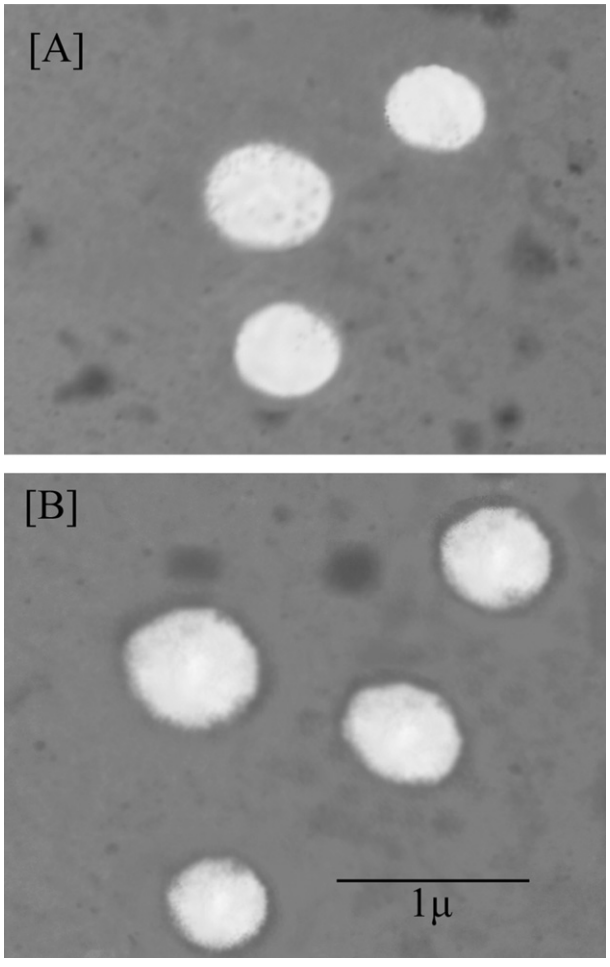

Fig. 1. Transmission electron micrographs of (A) plain liposomes and (B) HA complexed liposomes.

3.2. *In process stability*

In the present study antigen was encapsulated in novel carriers to enhance immunogenicity of antigen. It is therefore essential that its three-dimensional structural stability should be preserved in encapsulation process. To investigate structural stability of antigens SDS-PAGE analysis was performed. A prominent band of protein for pure as well as antigen from liposomes was detected in SDS-PAGE (Fig. 3). From analysis it could be concluded that the protein HBsAg and HA were not cleaved during the encapsulation process. Further the ratio of EIA to protein concentration was determined for to confirm structural integrity of HBsAg. The HBsAg from liposomal formulations showed an EIA/protein ratio of 0.92 ± 0.07 and 0.94 ± 0.11 for Lipo(HBsAg)-HA and Lipo(HBsAg), respectively. On the other hand plain antigen in PBS buffer shows EIA/protein ratio of 1.0 ± 0.1. This indicates that the antigenicity of antigen was not altered during formulation process.

3.3. *Haemagglutination assay and functional reactivity of HA protein*

The haemagglutination assay was used to examine whether the functional reactivity of HA protein was retained or not as it is the indicative of conformational integrity of the protein (Wang et al., 2006). A positive agglutination of RBC was observed with no significant difference between plain HA in buffer and liposomes coupled

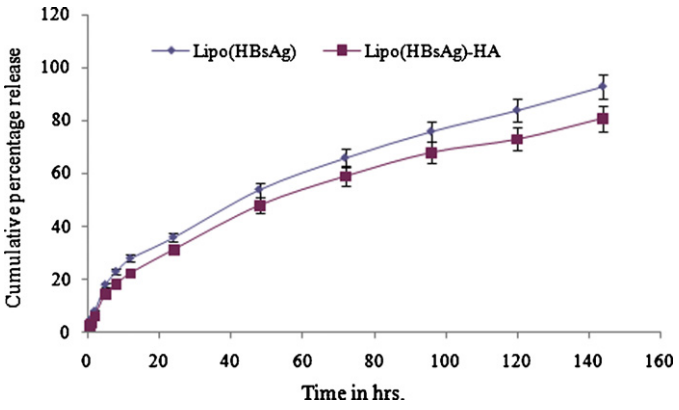

Fig. 2. *In vitro* cumulative release profile of various liposomal formulations.

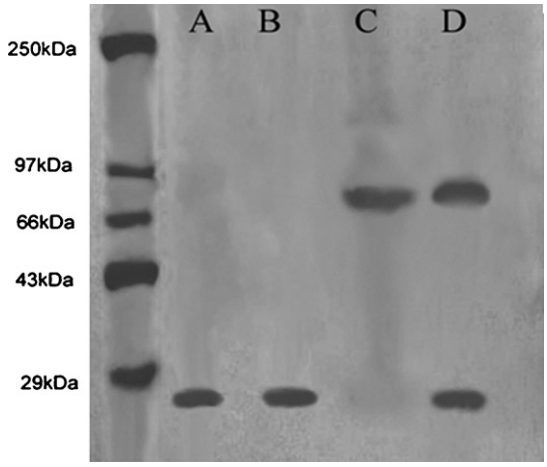

Fig. 3. Molecular characterization of antigen-(A) SDS-PAGE analysis-Lane A: pure HBsAg; Lane B: encapsulated HBsAg in liposome; Lane C: pure HA; Lane D: HA complexed liposomes formulation.

HA. Further, no agglutination was observed in control wells incubated with buffer or plain liposomes. The binding and agglutination capability with RBC is the characteristic of haemagglutinin protein and even the protein is named as due this property (Killian, 2008). On the basis of results obtained it may be postulated that the confirmation of the antigen had not altered during complexation onto the liposomes.

3.4. Fluorescent microscopy study

Fluorescent microscopy was performed in order to confirm disposition of carrier system in the nasal associated lymphoid tissues. The FITC labeled antigen HBsAg was used as marker. As per the characteristic binding property of HA to mucosal surface (Wu et al., 2004; Lakadamyali et al., 2004) the HA conjugated liposomal formulation was found to adhere to the mucosal surface to a greater extent as compared to plain liposomes (Fig. 4). Further, minimum localization of fluorescence was observed in FITC labeled antigen in PBS buffer. The M cells are the principal cells having highest concentration of receptor for HA (Fujimura et al., 2004; Kumlin et al., 2008). Subsequently, it is reasonable to suggest that they could be sampled primarily *via* M cells and further transported to sub-mucosal layer and this reflects their capacity to efficiently transport followed by presentation to underlying lymphoid tissues.

3.5. Immunological response

The *in vivo* immunogenocity of the designed vaccine formulation was evaluated by measuring systemic as well as mucosal immune response elicited following intranasal administration and compared with conventional liposome and plain antigen formulations. Alum adsorbed formulation of antigen administered intramuscularly was used as control for comparison purpose (protocol of immunization study has been described in Table 1).

The serum humoral immune response was measured in terms of anti-HBsAg IgG antibody titer and measured by ELISA at definite time intervals. The nasal administration of liposome elicited serum antibody response that was significantly higher, the highest antibody titer elicited by HA complexed liposomes. However, it was less than the response elicited by conventional alum adsorbed formulation on intramuscular administration (Fig. 5). Further the maximum antibody titer was obtained after 4 week of primary immunization and this provides the evidence of boosting on the immune response.

Mucosal immunity plays an important role in protection against pathogen that enters in the host *via* respiratory, gastrointestinal and urogenital route. The mucosal site is equipped with the organized mass of lymphoid tissue, the specialized immune inductive site that consists of M cells for antigen sampling, B cells, T cells, plasma cells, professional antigen presenting cell and draining lymphatic vesicles that are essential for induction and maintenance of immune response. In the case of nasal mucosal vaccine delivery uptake of antigen by nasal associated lymphoid tissue is prerequisite for the induction of immune response. The humoral mucosal immune response elicited by various liposomal formulations was measured by estimating the secretory IgA response in mucosal fluids. For the purpose nasal, vaginal and salivary secretions were collected after 6 week of primary immunization and measured for

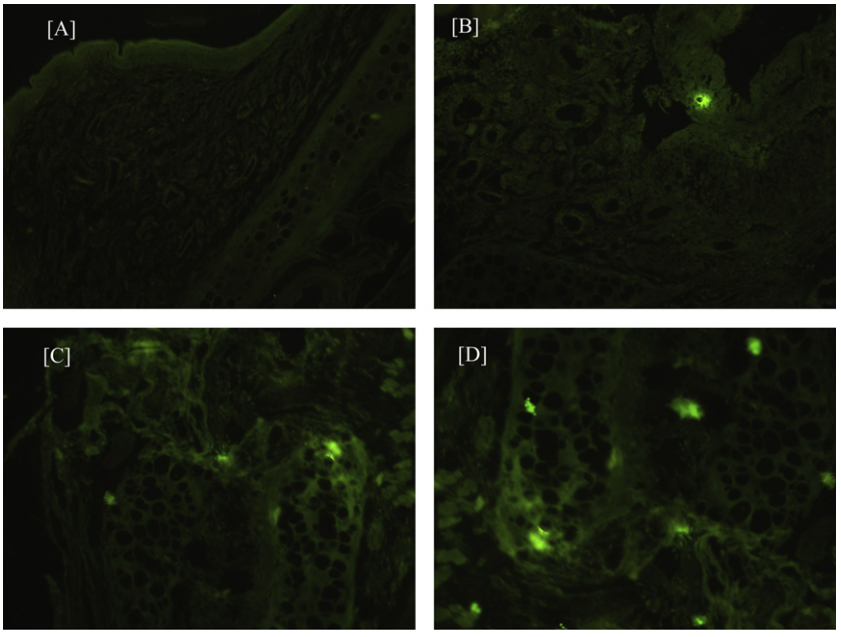

Fig. 4. Fluorescence micrographs of the nasal tissue of mice excised following administration of (A) control, (B) FITC-HBsAg solution; (C) FITC-HBsAg encapsulated in plain liposomes or (D) FITC-HBsAg encapsulated in HA complexed liposomes.

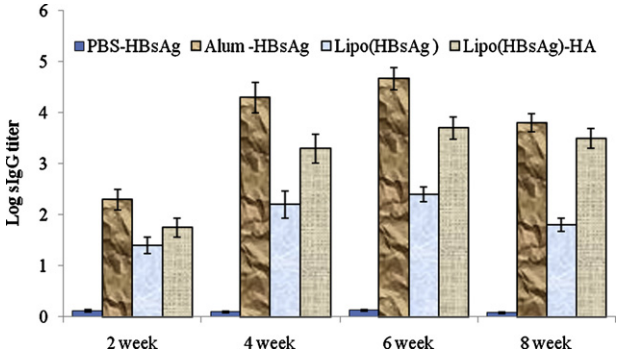

Fig. 5. Serum anti-HBsAg profile of mice immunized with different formulations. Values are expressed as mean ± S.D. ($n = 6$). The titer obtained after administration of various formulations comparable with titer recorded after alum-HBsAg administered intramuscularly ($p > 0.05$). The serum was collected after 2, 4, 6 and 8 weeks of primary immunizations.

anti-HBsAg level by ELISA. Fig. 6 depicts s-IgA level in various secretions of the body fluids and indicated that the highest mucosal immune response (in terms of anti-HBsAg IgA) was recorded for HA complexed liposomes (HA-Lipo-HBsAg), which was considerably higher than that elicited by plain liposomes and antigen solution in PBS buffer. Negligible mucosal immune response was observed in the case of alum adsorbed antigen administered intramuscularly. The results were in accordance with the previous finding that the systemic immunity is never transferred to mucosal site, furthermore the surface decoration of the carrier system with any of the molecules that enhances its interaction with mucosal cells (principally M cells) ultimately augments the immunization potential of the carrier (Gupta et al., 2006; Mishra et al., 2010; Shukla et al., 2010). Further, the liposomal formulations (both plain and HA complexed liposomes) following intranasal administration produced high level of secretory IgA in nasal, salivary and vaginal secretions indicating successful induction of mucosal immunity in all mucosal compartment. This occurs because common mucosal immune system immunization at one mucosal inductive site leads to immunity generation at distal mucosal effector site (Kutteh et al., 2001; Crowley-Nowick et al., 1997; Hamann et al., 1994). The results indicate that HA complexed liposomes induces both systemic and mucosal humoral immune response.

3.6. Cytokine response

Previous work on influenza virus and HA protein revealed that the HA protein on the surface of virus causes fusion of virus

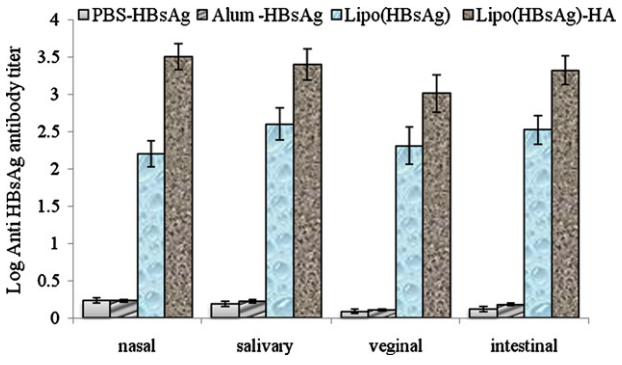

Fig. 6. sIgA level in mucosal secretions after intranasal immunization with HBsAg encapsulating liposomal formulations. The salivary, intestinal and vaginal secretions were collected after six week of first immunization. The differences in the antibody level was significant ($p < 0.05$) among Lipo(HBsAg), Lipo(HBsAg)-HA and Alum-HBsAg.

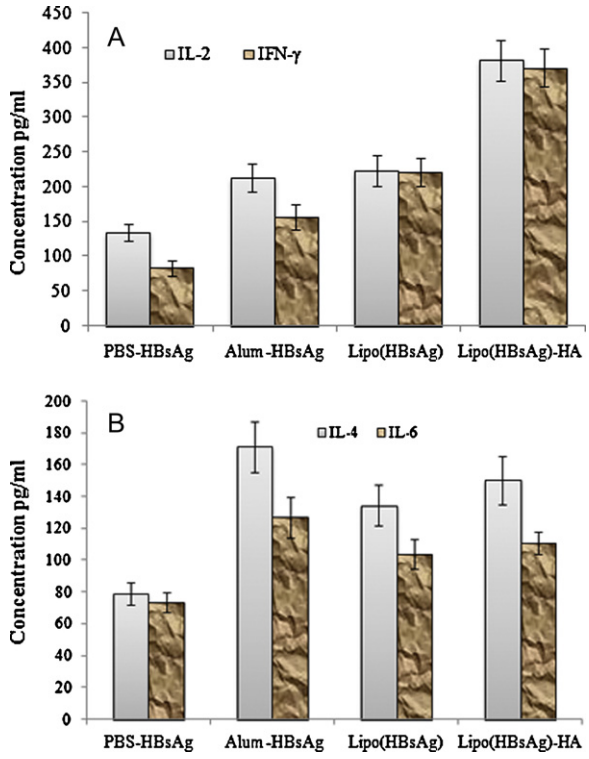

Fig. 7. Interferon-γ and interleukin (IL-2, IL-4, IL-6) levels in the spleen of mice immunized with various formulations. Values are expressed as mean ± S.D. ($n = 3$). The HA complexed liposomal formulations showed significant differences from alum-HBsAg and plain liposomal HBsAg.

envelop with endosomal membrane that ultimately release the encapsulated content (i.e., genetic material of virus) in cytosole (Sammalkorpi and Lazaridis, 2007; Lakadamyali et al., 2004; Lee et al., 2006). Therefore, the surface immobilization of the HA on to the surface of lipid membrane of liposomes is expected to mimic the same intracellular pathway as the virus does.

Further, subsequent to internalization of antigen carrier system by APC (*via* the process of endocytosis or phagocytosis) it is the characteristics of carrier system (or any constituent of carrier system) that ultimately decides the fate of immune response generated. The processing of carrier system *via* endocytic pathway ultimately leads to generation of antigen specific humoral immunity that helps in elimination of extracellular pathogens. Alternatively, processing of antigen by cytosolic pathway could be possible by the release of endosomal content into the cytosole by means of a pH sensitive fusogenic moiety. This ultimately leads to stimulation of cytotoxic T cells, i.e., cell mediated immunity which helps in elimination of intracellular pathogen. Being intracellular pathogen cell mediated immunity is the prime requirement for elimination of hepatitis B virus.

The presence and concentration of cytokines gives indirect evidence that which kind of immunity is primarily involved in the immune response generation. Indeed, the Th1 based cytokines IL-2 and IFN-γ are evidence of strong cell mediated immunity and Th-2 based cytokines, i.e., IL-4 and IL-6 are evidence of high level of humoral immunity. The cytokine response elicited by various liposomal formulations is given in Fig. 7. The data clearly depicts that the CMI response was dominated in the case of HA complexed liposomal formulation as it produced highest level of IL-2 and IFN-γ which is significantly higher than that elicited by other formulations (i.e., plain liposomes and alum adsorbed antigen formulation). On the other hand alum adsorbed formulation elicited highest level of IL-4 and IL-6 indicative of humoral response. The result confirms

the earlier findings that the alum is basically Th-2 based adjuvant and cannot elicit CMI (Tiwari et al., 2009), whereas incorporation of pH sensitive fusogenic molecule within the liposomal constituent enhances the level of cell mediated immunity against the antigen owing to cytosolic release of encapsulated content (Khatri et al., 2008).

4. Conclusion

It is clear from the results that recombinant haemagglutinin protein can be successfully complexed onto the surface of liposomes. The study demonstrates that significant binding to mucosal M cells followed by production of mucosal (locally and in distal mucosal site) as well as systemic immune (both humoral and cellular) response can be achieved with HA complexed liposomal formulation. The complexation of viral protein HA on to the surface of liposomes thus seems to be a prospective approach for development of recombinant antigen based liposomal mucosal vaccines. It may have potential to be employed for drug delivery and gene therapy *via* mucosal route. The intracellular localization as well as the FACS analysis revealing effect of cellular uptake on HA complexation on the liposomes and the immunogenicity of the surface immobilized protein are under study.

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References

Alpar, H.O., Bowen, J.C., Brown, M.R.W., 1992. Effectiveness of liposomes as adjuvants of orally and nasally administered tetanus toxoid. Int. J. Pharm. 88, 335–344.
Anabousi, S., Laue, M., Lehr, C.M., Bakowsky, U., Ehrhardt, C., 2005. Assessing transferrin modification of liposomes by atomic force microscopy and transmission electron microscopy. Eur. J. Pharm. Biopharm. 60, 295–303.
Aramaki, Y., Fujii, Y., Yachi, K., Kikuchi, H., Tsuchiya, S., 1994. Activation of systemic and mucosal immune response following nasal administration of liposomes. Vaccine 12, 1241–1245.
Bogdanov, A.A., Klibanov, A.L., Torchilin, V.P., 1988. Protein immobilization on the surface of liposomes via carbodiimide activation in the presence of N-hydroxysulfosuccinimide. FEBS Lett. 231, 381–384.
Crowley-Nowick, P.A., Bell, M.C., Brockwell, R., Edwards, R.P., Chen, S., Partridge, E.E., Mestecky, J., 1997. Rectal immunization for induction of specific antibody in the genital tract of women. J. Clin. Immunol. 17, 370–379.
Debin, A., Kravtzoff, R., Santiago, J.V., Cazales, L., Sperandio, S., Melber, K., et al., 2002. Intranasal immunization with recombinant antigens associated with new cationic particles induces strong mucosal as well as systemic antibody and CTL responses. Vaccine 20, 2752–2763.
Elson, C.O., Ealding, W., Lefkowitz, J., 1984. A lavage technique following repeated measurement of IgA antibody in mouse intestinal secretions. J. Immunol. Methods 67, 101–108.
Fry, D.W., White, J.C., Goldman, I.D., 1978. Rapid separation of low molecular weight solutes from liposomes without dilution. Anal. Biochem. 90, 809–815.
Fujii, Y., Aramaki, Y., Hara, T., Yachi, K., Kikuchiand, H., Tsuchiya, S., 1993. Enhancement of systemic and mucosal immune responses following oral administration of liposomes. Immunol. Lett. 36, 65–70.
Fujimura, Y., Takeda, M., Ikai, H., Haruma, K., Akisada, T., Harada, T., Sakai, T., Ohuchi, M., 2004. The role of M cells of human nasopharyngeal lymphoid tissue in influenza virus sampling. Virchows Arch. 444, 36–42.
Gregoriadis, G., Tan, L., Ben-Ahmeida, E.T.S., Jennings, R., 1992. Liposomes enhance the immunogenicity of reconstituted influenza virus A/PR/8 envelopes and the formation of protective antibody by influenza virus A/Sichuan/87 (H3N2) surface antigen. Vaccine 10, 747–753.
Gupta, P.N., Mahor, S., Rawat, A., Khatri, K., Goyal, A., Vyas, S.P., 2006. Lectin anchored stabilized biodegradable nanoparticles for oral immunization 1. Development and in vitro evaluation. Int. J. Pharm. 318, 163–173.
Hamann, A., Andrew, D.P., Jablonski Westrich, D., Holzmann, B., Butcher, E.C., 1994. Role of α4 integrins in lymphocyte homing to mucosal tissues in vivo. J. Immunol. 152, 3282–3293.
Hermanson, G.T., 1996. Bioconjugate Techniques. Academic Press, San Diego, CA.
Holmgren, J., Czerkinsky, C., Eriksson, K., Mharandi, A., 2003. Mucosal immunization and adjuvants: a brief overview of recent advances and challenges. Vaccine 21 (Suppl. 2), 589–595.
Kemble, G., Greenberg, H., 2003. Novel generations of influenza vaccines. Vaccine 21, 1789–1795.
Khatri, K., Goyal, A.K., Gupta, P.N., Mishra, N., Mehta, A., Vyas, S.P., 2008. Surface modified liposomes for nasal delivery of DNA vaccine. Vaccine 26, 2225–2233.
Khatri, K., Rawat, A., Mahor, S., Gupta, P.N., Vyas, S.P., 2005. Hepatitis B surface protein docked vesicular carrier for site specific delivery to liver. J. Drug Target. 13, 359–366.
Killian, M.L., 2008. Hemagglutination assay for the avian influenza virus. Methods Mol. Biol. 436, 47–52.
Kumlin, U., Olofsson, S., Dimock, K., Arnberg, K., 2008. Sialic acid tissue distribution and influenza virus tropism. Influenza and Other Respiratory Viruses 2, 147–154.
Kutteh, W.H., Kantele, A., Moldoveanu, Z., Crowley-Nowick, P.A., Mestecky, J., 2001. Induction of specific immune responses in the genital tract of women after oral or rectal immunization and rectal boosting with Salmonella typhi Ty 21a vaccine. J. Reprod. Immunol. 52, 61–75.
Lakadamyali, M., Rust, M.J., Zhuang, X., 2004. Endocytosis of influenza viruses. Microbes Infect. 6, 929–936.
Lee, J.H., Goulian, M., Boder, E.T., Lee, J.H., Goulian, M., Boder, E.T., 2006. Autocatalytic activation of influenza hemagglutinin. J. Mol. Biol. 364, 275–282.
Lemoine, D., Francotte, M., Preat, V., 1998. Nasal vaccines from fundamental concepts to vaccine development. Pharm. Sci. 8, 5–18.
Mishra, N., Tiwari, S., Vaidya, B., Agrawal, G.P., Vyas, S.P., 2010. Lectin anchored PLGA nanoparticles for oral mucosal immunization against hepatitis B. J. Drug Target., 1–12 (early online).
Munoz, M., Garcia, M., Reig, F., Alsina, M.A., Haro, I., 1998. Physico-chemical characterization of liposomes with covalently attached hepatitis A VP3(101–121) synthetic peptide. Analyst 123, 2223–2228.
Munoza, M., Sospedra, P., Gómara, M.J., Mestres, C., Haro, I., 2004. The covalent coupling of HAV-VP3 (110–121) synthetic peptide to liposomes: physicochemical studies. Int. J. Pharm. 269, 177–184.
Mutsch, M., Zhou, W., Rhodes, P., Bopp, M., Chen, R.T., Linder, T., Spyr, C., Steffen, R., 2004. Use of the inactivated intranasal influenza vaccine and the risk of Bell's palsy in Switzerland. N. Engl. J. Med. 350, 896–903.
Nakane, A., Numata, A., Minagawa, T., 1992. Endogenous tumor necrosis factor, interleukin-6, and gamma interferon levels during Listeria monocytogenes infection in mice. Infect. Immun. 60, 523–528.
Neutra, M.R., Kozlowski, P.A., 2006. Mucosal vaccines: the promise and the challenge. Nat. Rev. Immunol. 6, 148–158.
Nossal, J.V., 2000. The global alliance for vaccines and immunization—a millennial challenge. Nat. Immunol., 5–8.
Ogra, P.L., Faden, H., Welliver, R.C., 2001. Vaccination strategies for mucosal immune responses. Clin. Microbiol. Rev. 14, 430–445.
Olde-Damink, L.H.H., Dijkstra, P.J., Van Luyn, M.J.A., Van Wachem, P.B., Nieuwenhuis, P., Feijen, J., 1996. *In vitro* degradation of dermal sheep collagen cross-linked using a water-soluble carbodiimide. Biomaterials 17, 679–684.
Perrie, Y., Gregoriadis, G., 2000. Liposome-entrapped plasmid DNA: characterisation studies. Biochim. Biophys. Acta 1475, 125–132.
Rai, S., Paliwal, R., Vaidya, B., Khatri, K., Goyal, A.K., Gupta, P.N., Vyas, S.P., 2008. Targeted delivery of doxorubicin via estrone-appended liposomes. J. Drug Target. 16, 455–463.
Rescigno, M., Urbano, M., Valzasina, B., Francolini, M., Rotta, G., Bonasio, R., Granucci, F., Kraehenbuhl, J.P., Ricciardi-Castagnoli, P., 2001. Dendritic cells express tight junction proteins and penetrate gut epithelial monolayers to sample bacteria. Nat. Immunol. 2, 361–367.
Ryan, E.J., Daly, L.M., Mills, K.H.G., 2001. Immunomodulators and delivery systems for vaccination by mucosal routes. Trends Biotechnol. 19, 293–304.
Sammalkorpi, M., Lazaridis, T., 2007. Configuration of influenza hemagglutinin fusion peptide monomers and oligomers in membranes. Biochim. Biophys. Acta 1768, 30–38.
Sazoka, F.C., Papahadjopoulos, D., 1978. Procedure for the preparation of liposomes with large aqueous space and high capture by reverse-phase evaporation. Proc. Natl. Acad. Sci. U.S.A. 75, 4194–4198.
Shi, L., Caulfield, M.J., Chern, R.T., Wilson, R.A., Sanyal, G., Volkin, D.B., 2002. Pharmaceutical and immunological evaluation of a single-shot hepatitis B vaccine formulated with PLGA microspheres. J. Pharm. Sci. 91, 1019–1035.
Shukla, A., Katare, O.P., Singh, B., Vyas, S.P., 2010. M-cell targeted delivery of recombinant hepatitis B surface antigen using cholera toxin B subunit conjugated bilosomes. Int. J. Pharm. 385, 47–52.
Singh, M., Li, X., McGhee, J.P., Zamb, T., Koff, W., Wang, C.Y., O'Hagan, D.T., 1997. Controlled release microparticles as a single dose Hepatitis B vaccine: evaluation of immunogenicity in mice. Vaccine 15, 475–481.
Singh, P., Prabakaran, D., Jain, S., Mishra, V., Jaganathan, K.S., Vyas, S.P., 2004. Cholera toxin B subunit conjugated bile salt stabilized vesicles (bilosomes) for oral immunization. Int. J. Pharm. 278, 379–390.
Singh, S.K., Bisen, P.S., 2006. Adjuvanticity of stealth liposomes on the immunogenicity of synthetic gp41 epitope of HIV-1. Vaccine 24, 4161–4166.
Stevens, J., Blixt, O., Glaser, L., Taubenberger, J.K., Palese, P., Paulson, J.C., Wilson, I.A., 2006. Glycan microarray analysis of the hemagglutinins from modern and pandemic influenza viruses reveals different receptor specificities. J. Mol. Biol. 355, 1143–1155.
Tardi, P.G., Swartz, E.N., Harasym, T.O., Cullis, P.R., Bally, M.B., 1997. An immune response to ovalbumin covalently coupled to liposomes is prevented when the liposomes used contain doxorubicin. J. Immunol. Methods 210, 137–148.

Tiwari, S., Goyal, A.K., Mishra, N., Khatri, K., Vaidya, B., Mehta, A., Wu, Y., Vyas, S.P., 2009. Development and characterization of novel carrier gel core liposomes based transmission blocking malaria vaccine. J. Control. Release 140, 157–165.

Tyrer, P., Foxwell, A.R., Cripps, A.W., Apicella, M.A., Kyd, J.M., 2006. Microbial pattern recognition receptors mediate M-cell uptake of a gram-negative bacterium. Infect. Immun. 74, 625–631.

Wang, K., Holtz, K.M., Anderson, K., Chubet, R., Mahmoud, W., Cox, M.M.J., 2006. Expression and purification of an influenza hemagglutinin-one step closer to a recombinant protein-based influenza vaccine. Vaccine 24, 2176–2185.

Wenxin Wu, Gillian, M., 2004. AirBinding of influenza viruses to sialic acids: reassortant viruses with A/NWS/33 hemagglutinin bind to a2,8-linked sialic acid. Virology 325, 340–350.

Wu, H., Williams, K., Singh, S.R., Scissum-Gunn, K., Singh, N.K., Teresa, D., Giambrone, J.J., 2009. Integrity of a recombinant hemagglutinin protein of an avian influenza virus. Biotechnol. Lett. 31, 1511–1517.

Yuki, Y., Kiyono, H., 2003. New generation of mucosal adjuvants for the induction of protective immunity. Rev. Med. Virol. 13, 293–310.