JPP Journal of Pharmacy and Pharmacology

JPP 2006, 58: 303–310

Received June 22, 2005
Accepted November 28, 2005
DOI 10.1211/jpp.58.3.0003
ISSN 0022-3573

Chitosan nanoparticles encapsulated vesicular systems for oral immunization: preparation, in-vitro and in-vivo characterization

Sanyog Jain, Rakesh Kumar Sharma and S. P. Vyas

Abstract

BSA-loaded chitosan nanoparticles were prepared and encapsulated in vesicles (liposomes and niosomes) to make them acid resistant upon oral administration. Prepared systems were characterized in-vitro for shape, size, entrapment efficiency and stability in simulated gastric fluid (SGF, pH 1.2) and simulated intestinal fluid (SIF, pH 7.5). The immune stimulating activity was studied by measuring serum IgG titre and secretory IgA (sIgA) levels in mucosal secretions following oral administration of various formulations in albino rats. Significantly higher ($P < 0.05$) serum IgG titres were achieved following oral administration of novel nanoparticulate vesicular formulations as compared with unmodified chitosan nanoparticles. Further, high sIgA levels in mucosal secretions advocated a possible application of chitosan nanoparticle encapsulated in vesicles as an oral vaccine delivery carrier-adjuvant system.

Introduction

Traditional needle-based, invasive, parenteral vaccination methods suffer from various drawbacks, like the need for trained personnel to administer vaccines, expense, patient inconvenience and the associated risk of needle-borne infections (AIDS, hepatitis, etc.) due to the use of contaminated needles. Further, parenterally administered vaccines mainly stimulate a systemic response and antibodies generated in this manner do not always reach the mucosal surfaces, which is the predominant entry site for most infectious pathogens. Mucosal immunization provides the first line of immunological defence (i.e., induction of secretory IgA (sIgA)) that prevents the attachment of infectious pathogens to the mucosa, thereby preventing any possible damage to the host (Medina & Guzman 2000).

Oral immunization offers the safest and most convenient way to induce mucosal immunity. However, it has not been successful to date because of the inefficient presentation of antigens to processing sites and their instability in the gastrointestinal tract. Orally administered antigens are mostly degraded by the acidic environment and inactivating enzymes of the stomach before reaching the M-cells of the Peyer's patches in the gastrointestinal tract. Also, sufficient uptake of the antigen by the Peyer's patches could not be achieved, so an immune reaction may not be elicited. To circumvent these problems, encapsulation of antigen and delivering it in a safer way has been proposed over the years. Among the various proposed delivery vehicles, vesicular systems (liposomes and niosomes) offer a number of potential advantages and have attracted considerable interest as mucosal delivery systems (Michalek et al 1989, 1992; Jackson et al 1990; Rentel et al 1999; Conacher et al 2001). However, the susceptibility of conventional vesicles (both liposomes and niosomes) to bile-salt-caused dissolution and enzymatic degradation in the gastrointestinal tract remains the main barrier for their effective oral delivery and warrants the development of some alternative versions of vesicles capable of protecting the contents to make them available intact at the site of their capture and uptake. In our laboratory, polysaccharide-coated vesicles (liposomes and niosomes) that remain stable in the gastrointestinal tract have been developed for oral immunization (Venkatesan & Vyas 2000; Jain et al 2005). We have also reported cholera toxin B subunit (CTB)-conjugated and bile-salt-stabilized niosomal vesicles (bilosomes) for protective and targeted delivery of antigen by the oral route (Singh et al 2004).

Chitosan, a natural polymer obtained by alkaline deacetylation of chitin, is cheap, non-toxic, biocompatible and biodegradable. It also serves as an excellent candidate for vaccine

Division of Radiopharmaceuticals, Nano Tech and Stem Cell Research, Institute of Nuclear Medicine and Allied Sciences, Defence Research and Development Organization, Brig. S. K. Mazumdar Road, Timarpur, Delhi-54, India

Sanyog Jain, Rakesh Kumar Sharma

Drug Delivery Research Laboratory, Department of Pharmaceutical Sciences, Dr Harisingh Gour University, Sagar [M.P.] 470 003, India

S. P. Vyas

Correspondence: S. Jain, Division of Radiopharmaceuticals, Nano Tech and Stem Cell Research, Institute of Nuclear Medicine and Allied Sciences, Defence Research and Development Organization, Brig. S. K. Mazumdar Road, Timarpur, Delhi-54, India. E-mail: sanyogjain@rediffmail.com

delivery because of its bioadhesiveness, high protein-binding efficiency and absorption-enhancement ability. Due to its polycationic nature it readily adheres to negatively charged surfaces, such as mucus and proteins, and promotes drug absorption (Illum 1998; Janes et al 2001; Thanou et al 2001; van der Lubben et al 2001; Hejazi & Amiji 2003). However, despite the excellent mucoadhesion and absorption-enhancement properties, oral vaccine delivery using chitosan-based formulations suffers from the high solubility of chitosan in the acidic pH of the stomach. It has been reported that in acidic medium (pH 1.2–2.0), tetracycline-loaded chitosan microspheres dissolved and the entire entrapped drug was released instantaneously, while 60% of the entrapped drug was released in 2 h when the pH of the medium was raised to 3.5 (Hejazi & Amiji 2002).

To overcome this drawback, we propose here the encapsulation of nanoparticles within lipid vesicular systems (liposomes and niosomes). Though phospholipids or surfactants in vesicular form are thought to be unstable in the gastrointestinal environment, it has been reported that gastric lipases do not hydrolyze phospholipids or lipoidal surfactants. The digestion of these lipids takes place mainly in the small intestine by pancreatic lipase, colipase, phospholipase A2 and cholesterol esterase, and by the action of bile salts (Thomson et al 1993). Thus, vesicular systems may apparently provide protection to chitosan nanoparticles in the stomach, while in the intestine the particles will be released from the vesicles, which ultimately may promote the absorption of loaded antigen through M-cells of the Peyer's patches due to its bioadhesive nature.

In this study, chitosan nanoparticles loaded with model antigen bovine serum albumin (BSA) were prepared and characterized. They were further encapsulated in liposomes and niosomes and the immunological response following their oral administration was measured.

Materials and Methods

Materials

Chitosan (practical grade, 75–85% deacetylated, MW 150 kDa) was obtained as a kind gift from M/s, Panacea Biotec Ltd (India). Soya phosphatidylcholine (PC), cholesterol, Sephadex G-100, BSA, Triton X-100, anti-rat IgA, and horse radish peroxidase (HRP)-labelled anti-rat IgG and anti-rat IgA were purchased from Sigma Chemicals Co. (USA). Surfactants; sorbitol mono stearate (Span 60) and polysorbates (Tween 20 and Tween 80) and glutaraldehyde were procured from Fluka (Switzerland). Substrate tetramethyl benzidine-hydrogen peroxide (TMB-H_2O_2) and bicinchoninic acid (BCA) protein estimation kit were obtained from Genei Bangalore (India). All other chemicals and solvents used were purchased from local suppliers and were of analytical grade unless mentioned.

Preparation of chitosan nanoparticles

Chitosan nanoparticles (CSP) were prepared by the coacervation/precipitation method, using sodium sulfate as precipitant, following the method described by Berthold et al (1996) and Tian & Groves (1999), with appropriate modifications as per laboratory setup. Chitosan was dissolved in an aqueous solution of acetic acid (1% v/v) containing 1% v/v Tween 80 to produce a 0.25% w/v solution of chitosan. A solution of sodium sulfate in distilled water (20% w/v) was added dropwise to 100 mL chitosan solution with sonication using a probe sonicator (Soniweld, India) operated at 50% frequency. Sonication was continued for 15 min after the addition of sodium sulfate, followed by mechanical stirring at 1000 rev min^{-1} for 30 min. Glutaraldehyde solution (25%, 1.0 mL) was added and stirring was continued for another hour, after which cross-linking was stopped by addition of sodium meta-bisulfite solution (12% w/v, 20 mL). The nanoparticles were purified by centrifugation at 5000 rev min^{-1} for 15 min and washing twice with double-distilled water. The purified nanoparticles then were lyophilized (Vacuubrand; Heto Dry Winner, Wertheim, Germany).

Optimization of sodium sulfate concentration

The concentration of precipitating agent for the formation of nanoparticles depends upon the molecular weight of the chitosan used. To optimize the sodium sulfate concentration, a solution of sodium sulfate (20% w/v) was added dropwise during the precipitation step to a final sodium sulfate concentration range of 0.1–1.0% w/v and the formation of nanoparticles was monitored by turbidity, examined by transmittance measurement at 500 nm. Percent transmittance was plotted graphically against the final concentration of sodium sulfate and the optimum concentration was determined from the graph at the point after which no significant change was observed in transmittance upon increasing the sodium sulfate concentration.

Antigen loading

Antigen was surface adsorbed onto the preformed nanoparticles following the method reported in the literature (Berthold et al 1996; Tian & Groves 1999) with appropriate modifications. BSA solutions in phosphate-buffered saline (PBS, pH 7.4) were added in different concentrations to 2% w/v dispersion of chitosan nanoparticles in PBS (pH 7.4). The mixture was shaken at room temperature for 3 h using a wrist action shaker (Yorco, India). BSA solution without chitosan nanoparticles was used as control. After 3 h the samples were centrifuged at 7000 rev min^{-1} for 10 min and the supernatants recovered were analysed for the protein content using the BCA method. The amount of loaded protein was calculated as the difference between results from the loaded media and from the control solution.

In-vitro characterization

Shape, size and zeta-potential

The shape and morphology was investigated by transmission electron microscopy (TEM) (Philips, Japan). Particle size and zeta-potential of the nanoparticles before and after antigen loading were determined using a particle size analyser (CILAS, 1064, France) and zetameter (Zetasizer 3000 HS; Malvern Instruments Co., UK), respectively.

In-vitro antigen release

About 50 mg of the prepared nanoparticles were dispersed in 10 mL PBS (pH 7.4) and incubated at 37 ± 2°C with constant shaking in a metabolic shaking bath (Indian equipment

corporation, Bombay). After appropriate time intervals the samples were centrifuged at 7000 rev min^{-1} for 10 min and supernatant (1 mL) was taken for protein assay using the BCA kit. The removed supernatant was replaced with fresh PBS and the process was repeated for up to 24 h. The cumulative amount of BSA released was calculated and plotted against time.

Preparation and characterization of chitosan nanoparticle-loaded vesicles

Liposomes composed of soya lecithin (PC) and niosomes composed of Span 60 were prepared by the reverse-phase. For niosomes evaporation technique as described by Szoka & Papahadjopoulos et al (1978) for liposomes and Kiwada et al (1985) for niosomes. Briefly, nanoparticles (5 mg of optimized formulation) were suspended in 2 mL PBS (pH 7.4) and used as the aqueous phase for the preparation of vesicles. Principal constitutive lipids of the vesicles (PC or Span 60) and cholesterol in different molar ratios were dissolved in 5 mL diethyl ether, followed by sonication with 2 mL aqueous phase (nanoparticle suspension) using a probe sonicator (Soniweld, India) for 5 min at 40% frequency. This led to the formation of a reverse (w/o type) emulsion. The reverse phase (organic solvent) of this emulsion was evaporated in a rotary flash evaporator at 37°C under reduced pressure (260–400 mmHg). The lipid gel so formed was collapsed and transformed into a fluid with continual vigorous mechanical agitation using a vortex mixer. To this, 3 mL of warm PBS (pH 7.4) was added to hydrate the vesicles. Unentrapped particles were removed by size exclusion chromatography by passing the niosomal suspension through a Sephadex G-100 mini column and centrifugation at 2000 rev min^{-1} for 3 min (Fry et al 1978; New 1990). The vesicles so obtained were observed under a phase contrast microscope (Leitz-Biomed, Germany) and transmission electron microscope (TEM; Philips, Japan) after negative staining. A fraction of 0.2 mL vesicles was lysed using minimum amount of Triton X-100 (0.5% v/v) and the liberated antigen (BSA) was estimated using the BCA method after suitable dilution to calculate the entrapment efficiency. Mean vesicle size was also determined using a particle size analyser (CILAS, 1064, France).

Stability in simulated gastric fluid and simulated intestinal fluid

This study involved the delivery of developed formulations by the oral route. Hence, it was necessary to study the stability of the formulations in simulated gastric fluid (SGF) and simulated intestinal fluid (SIF). SGF (pH 1.2) was prepared by dissolving 2 g of sodium chloride in water followed by addition of 3.2 g pepsin dissolved in 7 mL of concentrated hydrochloric acid. Water was added to make up the volume up to 1 L and the pH of the resultant solution was adjusted to 1.2. SIF (pH 7.5) was prepared by dissolving 6.8 g monobasic potassium phosphate in 250 mL water. Sodium hydroxide solution (190 mL, 0.2 N) was added along with 400 mL water, followed by the addition of 10 g pancreatin. To simulate the effect of bile, sodium deoxycholate in 20 mM concentration was also added. Water was then added to make up the volume upto 1 L and the pH was adjusted to 7.5 with 0.2 N sodium hydroxide solution. A 2-mL volume of the vesicular formulations or 10 mg of nanoparticles were added to 10 mL of SGF (pH 1.2) or SIF (pH 7.5) and were continuously shaken on a wrist action shaker in a water bath maintained at 37 ± 1°C for 2 h. The number of intact vesicles remaining and residual antigen content was determined.

Immunization experiments

Animals and inoculations

Wistar albino rats, 100–150 g, were used for in-vivo studies. The study was carried out under the guidelines compiled by CPCSEA (Committee for the Purpose of Control and Supervision of Experiments on Animal, Ministry of Culture, Government of India) and all study protocols were approved by the institutional Animal Ethics Committee. Rats were housed in groups of 5 and had free access to food and water. They were, however, deprived of food 3 h before oral immunization. For immunization, various formulations (alum-adsorbed BSA, unmodified antigen-loaded chitosan nanoparticles and nanoparticles encapsulated in liposomes/niosomes) in a dose equivalent to 100 μg of BSA were administered orally using a feeding cannula for three consecutive days followed by a booster dose 3 weeks after the first dose. The rats were also immunized using a single intramuscular injection of alum-adsorbed BSA followed by booster dose after 3 weeks to serve as a standard for comparison.

Sampling

Pre-immune samples of serum and mucosal secretions (saliva and intestinal lavage) were obtained on day 0 before immunization. Blood samples were collected from the retro-orbital plexus of rats at biweekly intervals for 8 weeks after immunization. Serum was obtained by centrifugation of blood samples and kept at −40°C until tested by ELISA. Mucosal secretions (salivary and intestinal) were collected 2 weeks and 4 weeks after primary immunization and tested for secretory IgA (sIgA) antibodies. For collection of saliva, rats were injected with 0.2 mL of a sterile solution of pilocarpine (10 mg mL^{-1}) intraperitoneally to increase salivary secretion. The rats began to salivate after approximately 20 min and the saliva was collected by capillary tube. Intestinal lavage was performed following the technique reported by Elson et al (1984). Briefly, four 0.5-mL doses of hyperosmotic lavage solution (NaCl 25 mM, Na_2SO_4 40 mM, KCl 10 mM, $NaHCO_3$ 20 mM and polyethylene glycol-MW 3350 48.5 mM) were administered intragastrically at 15-min intervals using a blunt-tipped feeding needle. Half an hour after the last dose of lavage solution, the rats were given 0.2 mL of pilocarpine (10 mg mL^{-1}) intraperitoneally to increase intestinal secretion. A discharge of intestinal contents occurred regularly over the next 20 min, which was collected carefully. These fluids were also stored at –40°C until analysed by ELISA for sIgA levels.

Analysis of immune response

Estimation of IgG levels in serum. Antibody responses in immunized rats were monitored using a microplate ELISA procedure. Microtitre plates (Nunc-Immuno Plate Fb 96;

Mexisorp, NUNC) were coated with 100 μL/well of 1% BSA solution in PBS (pH 7.4) and incubated overnight at 4°C. The plates were washed three times with PBS–Tween 20 (0.05% v/v) (PBST). The serum samples were serially diluted with PBS and 100 μL of each sample was added to each well of the coated ELISA plates. The plates were incubated for 1 h at room temperature and washed three times with PBST. A 100-μL volume of peroxidase-labelled goat anti-rat IgG was added to each well. The plates were covered and after incubation for 1 h at room temperature, washing was repeated. Substrate solution (TMB-H_2O_2, 100 μL/well) was added followed by addition of 50 μL of 2 N H_2SO_4 after 20 min to stop the reaction. The intensity of the developed colour was measured at 450 nm within 15 min using a plate reader (Labsystems, Finland). End-point titres were expressed as the log10 of the reciprocal of last dilution that gave an optical density (OD) at 450 nm above the OD of negative controls.

Estimation of sIgA levels in mucosal secretions. For estimation of sIgA levels, the same procedure as described above was followed. In this case, the wells were coated with goat anti-rat IgA (100 μL/well) and incubated overnight at 4°C. The plates were washed three times with PBST followed by addition of 100 μL/well blocking solution (1% w/v BSA) to prevent non-specific adsorption of antibodies. Appropriately diluted sample fluid (100 μL/well) was then added after the washing step. Subsequently, HRP-conjugated anti-rat IgA was added followed by addition of substrate (TMB-H_2O_2) and H_2SO_4 to develop colour and stop the reaction. Absorbance was read at 450 nm using a microtitre plate reader (Labsystems, Finland).

Statistical analysis

All the results were expressed as mean ± standard deviation (s.d.). One-way analysis of variance followed by post-hoc test (Tukey's test) was used to evaluate the effect of sodium sulfate concentration on the percentage transmittance for nanoparticle formation and also the effect of antigen to nanoparticle ratio (BSA:CSP w/w) on loading efficiency of the nanoparticles (n = 6). Analysis of serum IgG titres and sIgA titres in mucosal secretions, achieved after different time intervals following immunization using different formulations, was performed using two-way analysis of variance followed by post-hoc test (Tukey's test) (n = 5). Statistical significance was designated as $P < 0.05$.

Results and Discussion

Preparation of chitosan-based nanoparticles

Chitosan nanoparticles could be spontaneously formed as a result of ionic interaction between the positively charged amino groups on the chitosan molecules and negatively charged multivalent counter ions, such as phosphate, polyphosphate and sulfate. The attractive features of these techniques include the spontaneous formation of chitosan nanoparticles by use of a relatively mild process, the homogeneous and adjustable resultant particle size and there being no need for organic solvents (Berthold et al 1996; Tian & Groves 1999). The emulsion-based techniques, such as solvent extraction and solvent evaporation techniques (Thanoo et al 1992; Akbuga & Bergisadi 1996), use organic solvents that are problematic for the stability of antigens. For this reason, the sodium sulfate-based precipitation technique, reported by Berthold et al (1996) and Tian & Groves (1999), was chosen for nanoparticles formation in this study.

In the preparation method, chitosan was used at a concentration of 0.25% w/v. Higher concentrations were not practical for viscosity-related reasons. In a highly viscous solution, homogeneous distribution of sodium sulfate was not possible, and would have resulted in the formation of agglomerates. Addition of polysorbate 80 (Tween 80) was necessary to stabilize the suspension, without which the formation of agglomerates occurred.

Optimization of sodium sulfate concentration

For optimization of sodium sulfate concentration for the formation of nanoparticles, a solution of sodium sulfate (20% w/v) was added dropwise during the precipitation step to reach a final concentration of 0.1–1.0% w/v, and the change in transmittance at 500 nm was recorded. It was observed that on increasing the final sodium sulfate concentration, the percentage transmittance fell down steeply initially and, after attaining a minimum value at 0.4% w/v sodium sulfate, no significant change ($P > 0.05$) in transmittance was recorded on further increasing sodium sulfate concentration. Thus, 0.4% w/v was considered as the optimum sodium sulfate concentration for the formation of chitosan nanoparticles by this novel precipitation method.

Antigen loading on to chitosan nanoparticles

In this study, antigen BSA was adsorbed onto the previously manufactured empty chitosan nanoparticles. The extent of loading (adsorption) was dependent on the initial amount of antigen employed. Formulations using five different BSA-to-chitosan nanoparticle (CSP) weight ratios, 1:5, 2:5, 3:5, 4:5 and 5:5, were prepared and for identification purpose these formulations were coded as BSA-CSP1, BSA-CSP2, BSA-CSP3, BSA-CSP4 and BSA-CSP5, respectively. Antigen loading efficiency and zeta-potential were the basis for optimization of BSA-to-CSP ratio. It was observed that loading (adsorption) of BSA was rapid and efficient and could be attributed to ionic interaction between negatively charged BSA and cationic chitosan particles. On increasing the BSA-to-CSP ratio from 1:5 to 3:5, loading efficiency slightly decreased from 98.4 to 94.7% but this difference is minute and statistically insignificant ($P > 0.05$) (Table 1). However, the particle size tended to increase on increasing antigen amount, which might be ascribed to antigen adsorbed onto the particle surface aiding the weight and subsequent size of the particles. On increasing BSA-to-CSP ratio, the zeta-potential decreased, possibly due to the charge neutralization effect exerted by adsorption of anionic BSA on cationic chitosan particles' surface. At a BSA-to-CSP ratio of 3:5 w/w, particles having 94.7 ± 5.1% loading efficiency with 220 ± 35 nm average particle size and lowest positive zeta-potential (+5.6 ± 0.7 mV) were obtained (Table 1). On further

Table 1 Effect of antigen (BSA)-to-nanoparticle weight ratio on loading efficiency, particle size and zeta-potential of the particles

BSA-to-CSP ratio (w/w)	Loading efficiency (%)	Particle size (nm)	Zeta-potential (mV)
0:5	0	135 ± 15	+38.6 ± 2.1
1:5	98.4 ± 7.6	155 ± 22	+24.4 ± 1.9
2:5	96.1 ± 6.5	180 ± 32	+13.7 ± 1.2
3:5	94.7 ± 5.1	220 ± 35	+5.6 ± 0.7
4:5	76.3 ± 6.7	380 ± 42	−6.8 ± 0.8
5:5	60.8 ± 5.8	435 ± 51	−20.3 ± 1.7

Values are expressed as mean ± s.d., n = 6.

increasing the BSA-to-CSP ratio (i.e. 4:5 and 5:5), the colloidal suspension became aggregated and flocculated, and the overall charge of the nanoparticles was reversed and the zeta-potential became increasingly negative. At the same time, antigen loading was significantly reduced, which might be attributed to the saturation of cationic amino groups of the chitosan nanoparticles at higher antigen ratios. Moreover, the already adsorbed BSA might cause charge-induced repulsion and prevent further adsorption of BSA. At these higher ratios, due to aggregation and flocculation, particle size was also increased to a greater extent. On the basis of these observations, an antigen-to-CSP ratio of 3:5 w/w was considered to be optimum.

In-vitro characterization of chitosan nanoparticles

The shape and surface morphology of the nanoparticles was studied by transmission electron microscopy (TEM). The plain nanoparticles made exclusively of chitosan displayed a solid and consistent structure, whereas those loaded with antigen (BSA adsorbed onto their surface) exhibited a compact core surrounded by a thick but fluffy coating, presumably of BSA.

The in-vitro antigen release profiles of different BSA-loaded formulations were determined in PBS (pH 7.4) (Figure 1). Results demonstrated that formulations BSA-CSP1 to BSA-CSP3 showed a sustained release profile, although the release rate increased with an increase in BSA-to-CSP ratio in the formulation. An initial burst release followed by sustained release pattern was exhibited by formulations BSA-CSP4 and BSA-CSP5 with BSA to CSP ratio of 4:5 and 5:5 w/w, respectively (beyond the optimum ratio, 3:5 w/w). BSA was ionically adsorbed onto the nanoparticles' surface by forming an ion-pair between negatively charged BSA and positively charged amino groups of chitosan. As the concentration of BSA increased, especially beyond the optimum limit, the cationic amino groups of the chitosan molecules might have become saturated and were not available for ion-pair formation. Thus, on increasing BSA concentration, the ionic interaction with chitosan might have become weak and excess BSA might have resulted in initial burst release, although a slow release pattern was achieved after 4 h.

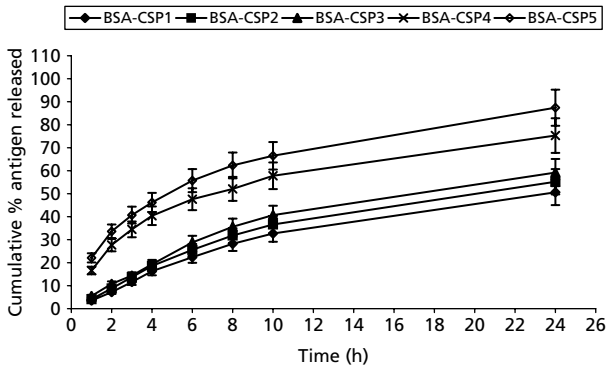

Figure 1 In-vitro antigen release profile of different chitosan nanoparticles formulations. Values are expressed as mean ± s.d., n = 6.

Chitosan nanoparticle-loaded vesicular systems

The prepared antigen-loaded chitosan nanoparticles were encapsulated within the vesicular carriers (liposomes and niosomes) to overcome the stability problem of unmodified particles in the acidic pH of the stomach. Optimization of the phospholipid/surfactant-to-cholesterol ratio was performed on the basis of microscopic evaluation and entrapment efficiency. Vesicular formulations with different PC/Span 60 and cholesterol ratios (9:1, 8:2, 7:3, 6:4 and 5:5 mole ratio) were prepared. The liposomal formulation (coded as Lip-CSP) having a PC-to-cholesterol mole ratio of 6:4 showed the maximum entrapment efficiency of 34.6 ± 3.1%, with a resultant mean vesicle size of 3.68 ± 0.32 μm (Table 2). On the other hand, the niosomal formulation (coded as Nio-CSP) that had a Span 60-to-cholesterol mole ratio of 7:3 exhibited a maximum entrapment efficiency of 35.7 ± 3.3% with an average vesicle size of 3.84 ± 0.35 μm (Table 2). These formulations were also found to be free of artifacts and aggregates on microscopic evaluation. Thus, based on these observations, these optimized formulations were selected for further characterization and other studies.

The shape and morphology of these nanoparticle encapsulated vesicular systems was determined by TEM. Both

Table 2 Entrapment efficiency and vesicle size of nanoparticle-loaded vesicles

	PC/Span 60-to-cholesterol molar ratio	Entrapment efficiency (%)	Mean vesicle size (μm)
Liposomes	9:1	28.2 ± 2.1	4.36 ± 0.38
	8:2	30.7 ± 2.8	4.17 ± 0.33
	7:3	32.9 ± 2.7	3.92 ± 0.30
	6:4	34.6 ± 3.1	3.68 ± 0.32
	5:5	31.5 ± 2.4	4.24 ± 0.41
Niosomes	9:1	30.4 ± 2.7	4.45 ± 0.41
	8:2	33.6 ± 3.1	4.12 ± 0.37
	7:3	35.7 ± 3.3	3.84 ± 0.35
	6:4	33.1 ± 3.2	4.21 ± 0.38
	5:5	29.5 ± 2.4	4.53 ± 0.42

Values are expressed as mean ± s.d., n = 6.

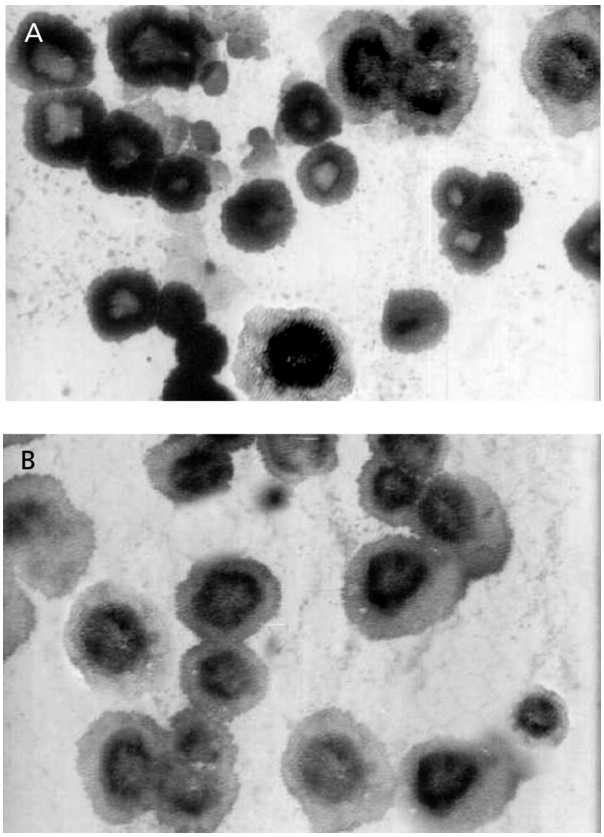

Figure 2 TEM image of chitosan nanoparticles encapsulated in liposomes (× 5500) (A) and niosomes (× 6000) (B).

vesicular formulations were spherical in shape and encapsulated nanoparticles could be clearly seen as dense black zones inside the vesicles in TEM images of the formulations (Figures 2A, B).

Stability in SGF and SIF

Since in this study the developed carrier systems were also used for oral delivery of antigens, their stability in SGF and SIF was realized to be of prime importance. The percentage of vesicles remaining, vis-à-vis percent antigen remaining after 2 h of incubation with SGF (pH 1.2) and SIF (pH 7.5), was determined (Table 3). The unmodified chitosan nanoparticles displayed a marked decrease in residual antigen content after incubation with SGF as only 36.4% residual antigen load was found on nanoparticles after incubation with SGF (pH 1.2) for 2 h. This may be attributed to the reported instability of chitosan at acidic pH where it tends to dissolve; this can be prevented by encapsulating the nanoparticles within vesicular systems. However, vesicular formulations also showed a significant decrease in vesicle count (vesicle loss of about 25–30%) after 2 h incubation with SGF, which may be attributed to aggregation of the vesicles in acidic medium. Despite this they could retain more than 65% of encapsulated content (chitosan nanoparticles) and offered significant protection against dissolution by the acidic pH of the stomach.

In SIF (pH 7.5) the nanoparticles remained stable and intact as a 76.9% residual intact antigen content was calculated. About 25% loss in antigen content might be attributed to the digestive effect of proteolytic enzymes present in SIF on the surface-adsorbed antigen on the nanoparticles, which can also be reduced by encapsulating the nanoparticles within vesicular formulations. In SIF, pancreatin lipase and bile salts causes destabilization/dissolution of the vesicles and released the encapsulated nanoparticles. This is reflected by a significantly lower vesicle count and antigen content of vesicles encapsulating chitosan nanoparticles (Lip-CSP and Nio-CSP) after incubation with SIF (Table 3).

Immunological response

Serum IgG levels

The serum IgG profile was determined for all experimental groups after 2, 4, 6 and 8 weeks of primary immunization to evaluate the systemic immune response elicited by the developed formulations (Figure 3). The serum IgG titre (log10) achieved after 4 weeks of oral immunization using native antigen (alum-adsorbed BSA), unmodified chitosan nanoparticles (BSA-CSP) and those encapsulated in liposomes (Lip-CSP) and niosomes (Nio-CSP) were 1.3 ± 0.2, 3.4 ± 0.4, 5.0 ± 0.6 and 5.3 ± 0.7, respectively. Unmodified nanoparticles (BSA-CSP) displayed a significantly lower IgG titre in serum after 4 weeks of oral immunization ($P < 0.05$), which can be attributed to the susceptibility of chitosan to the highly acidic pH of the stomach, resulting in dissolution of chitosan and subsequent exposure of the liberated antigens to the acidic pH and digestive enzymes. This limitation of

Table 3 Stability of developed formulations in SGF and SIF

Parameter(s)	In SGF (pH 1.2)			In SIF (pH 7.5)		
	CSP	Lip-CSP	Nio-CSP	CSP	Lip-CSP	Nio-CSP
Vesicle count ($\times 10^3$)						
Before incubation	—	37.8 ± 4.2	41.2 ± 4.1	—	37.8 ± 4.2	41.2 ± 4.1
After incubation	—	26.5 ± 3.4	29.8 ± 3.2	—	20.6 ± 2.7	23.4 ± 2.5
% Vesicles remaining	—	70.1 ± 6.3	72.3 ± 7.4	—	54.6 ± 6.5	56.8 ± 5.8
% Residual antigen content	36.4 ± 3.1	65.8 ± 4.2	67.4 ± 5.7	76.9 ± 6.3	52.7 ± 5.6	55.3 ± 6.3

Values are expressed as mean ± s.d., n = 6.

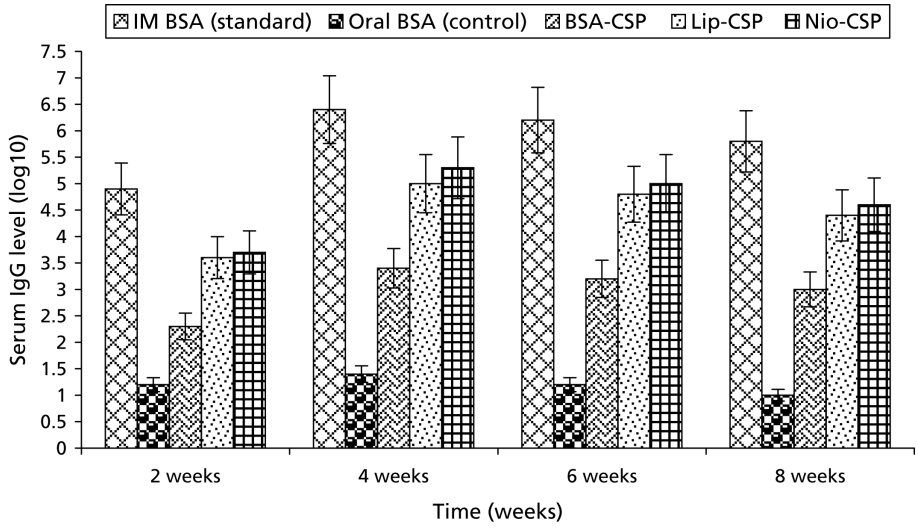

Figure 3 Serum IgG profile of rats immunized with different formulations. End-point titres were expressed as the log10 of the reciprocal of last dilution that gave an optical density (OD) at 450 nm above the OD of negative controls. Values are expressed as mean ± s.d., n = 5.

chitosan was circumvented by encapsulating the antigen-loaded nanoparticles within vesicles (liposomes and niosomes). It has been well established that gastric lipase does not hydrolyse phospholipids or surfactants and their digestion takes place in the small intestine by pancreatic lipases in the presence of bile salts. Therefore, encapsulation of chitosan nanoparticles into vesicles protects the chitosan particles from dissolution in the acidic pH of stomach, whereas in the small intestine they are liberated from the vesicles in their native unmodified form due to digestion of the lipoidal (phospholipid/surfactant) coat by the action of bile salts or pancreatin lipases. The liberated unmodified particles remained stable in the small intestine and might have been better absorbed through the M cells of Peyer's patches due to the strong mucoadhesive and absorption-enhancement properties of chitosan, which is reflected in the better immunological response produced by these novel systems. Thus, the enteric functioning can be implicated by using vesicular carrier systems.

Secretory IgA level in salivary and intestinal secretions

Figure 4 depicts sIgA levels in intestinal and salivary secretions of rats after 2 and 4 weeks of immunization using various formulations. It can be inferred from the data that the developed vesicular formulations more efficiently elicited a mucosal immune response (sIgA level) than unmodified nanoparticles. It has been well accepted that direct exposure of antigens to the mucosal membrane can cause a significant increase in antigen-specific secretory IgA response at intestinal and other mucosal surfaces. Compelling evidence has shown that stimulation of IgA precursor B cells in gut-associated lymphoid tissues (GALT) with orally administered antigens leads to the dissemination of B and T cells to mucosal effector tissues, such as the lamina propria regions of the intestinal, respiratory, genitourinary and various secretory glands for

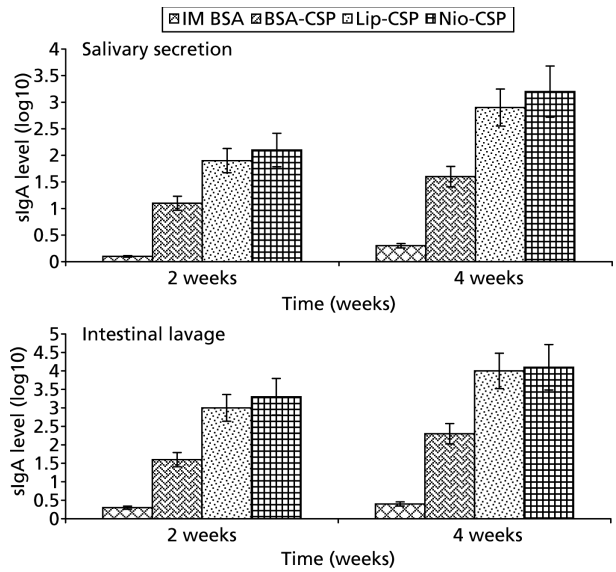

Figure 4 Secretory IgA (sIgA) level in intestinal and salivary secretions of rats immunized with different formulations. End-point titres were expressed as the log10 of the reciprocal of last dilution that gave an optical density (OD) at 450 nm above the OD of negative controls. Values are expressed as mean ± s.d., n = 5.

subsequent antigen-specific sIgA antibody responses (Mestecky 1987; McGhee et al 1989, 1992).

Negligible sIgA response (statistically insignificant, $P > 0.05$) was observed with intramuscularly administered alum-adsorbed BSA. This may be attributed to the fact that parenterally administered antigen lacks ability to stimulate the mucosal immune system. Thus the orally administered system can provide the additional advantage of mucosal immunity (i.e. neutralizing the antigen at first exposure).

Conclusion

It can be concluded from the results that the prepared nanoparticulate vesicular systems (i.e., liposome- and niosome-encapsulated antigen-loaded chitosan nanoparticles) could be used as effective carrier and adjuvant for non-invasive, non-parenteral mucosal immunization through the oral route. The proposed systems are capable of eliciting both systemic and mucosal immune responses and can prove as promising carrier systems for a number of antigens other than BSA. They can overcome the disadvantages of classical invasive methods of vaccination and are simple, economical, stable, painless and potentially safe. With the identification of such types of carrier systems that have potential to elicit systemic immunity following oral administration, a new way of low-cost administration of potential vaccines may be developed, particularly for use in developing countries. However, more studies concerning T lymphocyte proliferation assays and cytokine production should be conducted to demonstrate the possibility of a cell-mediated immune response elicited by these systems. The practical utility can only be realized following elaborative studies and clinical trials taken up especially with selected antigens.

References

Akbuga, J., Bergisadi, N. (1996) 5-Fluorouracil-loaded chitosan microspheres: preparation and release characteristics. *J. Microencapsul*. **13**: 161–168

Berthold, A., Cremer, K., Kreuter, J. (1996) Preparation and characterization of chitosan microspheres as drug carrier for prednisolone sodium phosphate as model for anti-inflammatory drugs. *J. Control. Release* **39**: 17–25

Conacher, M., Alexander, J., Brewer, J. M. (2001) Oral immunisation with peptide and protein antigens by formulation in lipid vesicles incorporating bile salts (bilosomes). *Vaccine* **19**: 2965–2974

Elson, C. O., Ealding, W., Lefkowitz, J. (1984) A lavage technique allowing 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

Hejazi, R., Amiji, M. (2002) Stomach-specific anti-H. pylori therapy I: preparation and characterization of tetracycline-loaded chitosan microspheres. *Int. J. Pharm*. **235**: 87–94

Hejazi, R., Amiji, M. (2003) Chitosan-based gastrointestinal delivery systems. *J. Control. Release* **89**: 151–165

Illum, L. (1998) Chitosan and its use as a pharmaceutical excipient. *Pharm. Res*. **15**: 1326–1331

Jackson, S., Mestecky, J., Childers, N. K., Michalek, S. M. (1990) Liposomes containing anti-idiotypic antibodies: an oral vaccine to induce protective secretory immune response specific for pathogens of mucosal surfaces. *Infect. Immun*. **58**: 1932–1936

Jain, S., Singh, P., Mishra, V., Vyas, S. P. (2005) Mannosylated niosomes as adjuvant-carrier system for oral genetic immunization against hepatitis B. *Immunol. Lett*. **101**: 41–49

Janes, K. A., Calvo, P., Alonso, M. J. (2001) Polysaccharide colloidal particles as delivery systems for macromolecules. *Adv. Drug Deliv. Rev*. **47**: 83–97

Kiwada, H., Fujisaki, Y., Yamada, S., Kato, Y. (1985) Application of synthetic alkyl glycoside vesicles as drug carriers. I. Preparation and physical properties. *Chem. Pharm. Bull*. **33**: 753–759

McGhee, J. R., Mestecky, J., Elson, C. D., Kiyono, H. (1989) Regulation of IgA synthesis and immune response by T cells and interleukins. *J. Clin. Immunol*. **9**: 175–199

McGhee, J. R., Mestecky, J., Dertzbaugh, M., Eldridge, J. H., Hirasawa, M., Kiyono, H. (1992) The mucosal immune system: from fundamental concepts to vaccine development. *Vaccine* **10**: 75–88

Medina, E., Guzman, C. A. (2000) Modulation of immune responses following antigen administration by mucosal route. *FEMS Immunol. Med. Microbiol*. **27**: 305–311

Mestecky, J. (1987) The common mucosal immune system and current strategies for induction of immune responses in external secretions. *J. Clin. Immunol*. **7**: 265–276

Michalek, S. M., Childers, N. K., Katz, J., Denys, F. R., Berry, A. K., Eldridge, J. H., McGhee, J. R., Curtiss, R. (1989) Liposomes as oral adjuvants. *Curr. Top. Microbiol. Immunol*. **146**: 51–58

Michalek, S. M., Childers, N. K., Katz, J., Dertzbaugh, M., Zhang, S., Russell, M. W., Macrina, F. L., Jackson, S., Mestecky, J. (1992) Liposomes and conjugate vaccines for antigen delivery and induction of mucosal immune responses. *Adv. Exp. Med. Biol*. **327**: 191–198

New, R. R. C. (1990) Introduction and preparation of liposomes. In: New, R. R. C. (ed.) *Liposomes: a practical approach*. Oxford University Press, Oxford, pp 1–104

Rentel, C. O., Bouwstra, J. A., Naisbett, B., Junginger, H. E. (1999) Niosomes as a novel peroral vaccine delivery system. *Int. J. Pharm*. **186**: 161–167

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

Szoka, F., Papahadjopoulos, D. (1978) Procedure for preparation of liposomes with large internal aqueous space and high capture by reverse-phase evaporation. *Proc. Natl Acad. Sci. USA* **75**: 4194–4198

Thanoo, B. C., Sunny, M. C., Jayakrishnan, A. (1992) Cross-linked chitosan microspheres: Preparation and evaluation as a matrix for the controlled release of pharmaceuticals. *J. Pharm. Pharmacol*. **44**: 283–286

Thanou, M., Verhoef, J. C., Junginger, H. E. (2001) Chitosan and its derivatives as intestinal absorption enhancers. *Adv. Drug Deliv. Rev*. **50** (Suppl. 1): S91–S101

Thomson, A. B., Schoeller, C., Keelan, M., Smith, L., Clandinin, M. T. (1993) Lipid absorption: passing through the unstirred layers, brush-border membrane and beyond. *Can. J. Physiol. Pharmacol*. **71**: 531–555

Tian, X. X., Groves, M. J. (1999) Formulation and biological activity of antineoplastic proteoglycans derived from Mycobacterium vaccae in chitosan nanoparticles. *J. Pharm. Pharmacol*. **51**: 151–157

van der Lubben, I. M., Verhoef, J. C., Borchard, G., Junginger, H. E. (2001) Chitosan for mucosal vaccination. *Adv. Drug Deliv. Rev*. **52**: 139–144

Venkatesan, N., Vyas, S. P. (2000) Polysaccharide coated liposomes for oral immunization: development and characterization. *Int. J. Pharm*. **203**: 169–177