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Research Article

Improved stability and immunological potential of tetanus toxoid containing surface engineered bilosomes following oral administration

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Abstract

The present study was designed with the objective to investigate the stability and potential of glucomannan-modified bilosomes (GM-bilosomes) in eliciting immune response following oral administration. GM-bilosomes exhibited desired quality attributes simultaneously maintaining the chemical and conformation stability of the tetanus toxoid (TT) entrapped in to freeze dried formulations. The GM-bilosomes exhibited excellent stability in different simulated biological fluids and sustained release profile up to 24 h. GM-bilosomes elicited significantly higher ($P < 0.05$) systemic immune response (serum IgG level) as compared to bilosomes, niosomes and alum adsorbed TT administered through oral route. More importantly, GM-bilosomes were found capable of inducing mucosal immune response, i.e. sIgA titre in salivary and intestinal secretions as well as cell mediated immune response (IL-2 and IFN- γ levels in spleen homogenate) which was not induced by i.m. TT, the conventional route of immunization. Conclusively, GM-bilosomes could be considered as a promising carrier and adjuvant system for oral mucosal immunization.

From the Clinical Editor: This team reports on the development and effects of a glucomannan-modified bilosome as an oral vaccine vector, using tetanus toxoid in the experiments. These GM-bilosomes not only elicited significantly higher systemic immune response as compared to bilosomes, niosomes and alum adsorbed orally administered TT, but also demonstrated mucosal immune response induction as well as cell mediated immune responses, which were not induced by the conventional route of immunization.

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Key words: Bilosomes; Vaccine delivery; Oral immunization; Mannosylation; Tetanus toxoid

A meticulous look towards the current approaches of immunization accentuates parenteral immunization as the principal route. It is worthy to note that almost 90% of the mammalian infections originate at mucosal surfaces while the conventional parenteral immunization elicits only systemic immune response not the mucosal immunity^{1,2} which is the prime need for immunization to be more successful against mucosal infections. Apart from this, high production cost, patient discomfort and risk of disease transmission³ are the other drawbacks of parenteral immunization which necessitate to find the potential alternatives. Oral immunization has been recognized as the most likely

alternative as it has been reported to induce systemic as well as mucosal immune response (predominant entry site for most infectious pathogens)⁴ and also provides more economical, convenient and safest route in contrast to conventional parenteral route.⁵

In spite of several advantages, oral immunization has not been so successful to date. Antigens on their way from oral administration to their pathological target are continuously challenged by multiple physiological barriers such as enzymatic degradation in harsh gastric environment and poor permeability across intestinal epithelium. Further relative weakness of gut immune system makes the oral immunization cumbersome.⁵ Thus the primary challenges in the field of oral immunization reside in the complete understanding of these barriers and development of novel strategies to circumvent them.

Issues pertinent to diverse challenges were taken into account and a variety of nanocarriers like polymeric micro/nanoparticles, liposomes, niosomes, and bilosomes have been reported with improved performance.^{6–12} Among the different nanocarriers, bilosomes seem to be the most promising due to high stability in

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gastrointestinal media¹³ nanometric size range,¹⁴ negative surface charge and hydrophobic nature.¹⁵ In addition, technical feasibility to scale up the vesicular systems is another advantage which can successfully convert the bilosomes into a clinical reality. In the quest to find more effective systems, advanced versions of vesicles are also reported in which vesicles have been decorated by anchoring suitable ligand for the variety of receptors *viz.* mannosyl, galactosyl, Fc and fibronectin, preferentially and abundantly present on the cell surface of antigen presenting cells (APCs) in mucosal linings.¹⁶ The efficacy of ligand anchorage in eliciting better immune response was also supported in our previous works in which both high systemic as well as mucosal immune responses were obtained upon oral administration of mannosylated niosomal formulations.^{1,2}

In the present study we report tetanus toxoid (TT) encapsulated mannosylated bilosomes for oral immunization. Glucosaminan (GM) was used as a ligand for targeted delivery to the mannosyl receptor of APCs. The polymeric nature of GM increases the surface functionality by increasing mannose molecules density on the surface which can enhance the uptake of mannosylated bilosomes by the APCs.^{17,18} Furthermore, the polymeric nature of GM can also provide stability against digestive enzymes.^{19–21} As far as the present literature is concerned this is the first report in which a polymer (GM) has been exploited for providing target specificity as well as additional stability to bilosomes. Therefore, in the present work three different formulations niosomes, bilosomes and mannosylated bilosomes containing TT, have been prepared and characterized for shape, size, stability, and immunological efficiency following oral administration.

Methods

Materials and reagents

TT (1850 Lf/mL, Batch No. TAM 046) was provided as generous gift sample by Panacea Biotec, India. Tetanus Toxoid (Adsorbed I.P.) (Serum Institute of India Ltd., Batch No. 01711017B) was purchased from local market. Secondary antibodies namely anti-mouse IgA (α -chain specific) peroxidase conjugate, anti-mouse IgG (γ -chain specific) peroxidase conjugate, 3,3',5,5'-tetramethylbenzidine (TMB), Nunc Immuno™ Maxisorb F96 well solid plates, Cholesterol (CH), sodium deoxycholate (Na-DOC), Span 80, coumarin-6, stearylamine, sodium dodecyl sulphate (SDS), Sephadex G 75, *N,N'*-Methylenebisacrylamide, acrylamide, ammonium persulphate (APS), Tetramethylethylenediamine (TEMED), and pilocarpine were purchased from Sigma, USA. Mouse IL-2 (catalog #KMC0021) and IFN- γ (catalog #KMC4021) ELISA kits were purchased from Invitrogen Corporation, USA. Bromophenol Blue, Coomassie Brilliant Blue G, Glycine, Ethyl dimethyl aminopropyl Diisopropyl Carbodiimide (EDC), β -mercaptoethanol, and Tris Buffer were obtained from Himedia, India. Konjac Glucosaminan (GM) Megazyme, Ireland, and distearyl phosphatidyl ethanolamine were obtained as kind gift samples from Lipoid, Germany. Chloroacetic Acid, pancreatin, pepsin were procured from Loba Chemie, India. Ultrapure water (LaboStar™ ultrapure water Systems, Germany) was used for

all the experiments. All other chemicals and reagents used were of analytical grade.

Synthesis of Glucosaminan-O-Carboxymethyl-Distearyl phosphatidyl ethanolamine (GM-OCM-DSPE) conjugate

The Synthesis of GM-OCM-DSPE conjugate was carried out in two steps. In the first step, GM was converted to intermediate, *o*-carboxy methylated glucosaminan (GM-OCM) by the previously reported method.²² GM-OCM was further converted to GM-OCM-DSPE in second step. Intermediate and final conjugates were further authenticated via Fourier transform infrared (FTIR) and nuclear magnetic resonance (NMR) spectroscopy. For interest, detailed synthesis scheme and spectra have been provided in the supplementary material.

Preparation of TT loaded niosomes and bilosomes

Different vesicular formulations encapsulating TT were prepared by thin film hydration method, as previously reported by our group.¹³ Niosomes were prepared by dissolving Span 80 and CH (2:1 mole ratio) along with stearyl amine (2% w/w of lipidic content) in chloroform and methanol (8:2 v/v) in a round bottom flask. Solvent removal under vacuum resulted in formation of thin film at the wall of round bottom flask. Subsequent hydration of the film with 10% w/v TT solution (10 mL) in water for 90 min at 37 °C resulted in formation of large multilamellar vesicles (LMVs). Sonication (3 cycles of 15 s with 1 min pulse off at 50 amplitude) in next step further converted LMVs to small unilamellar vesicles (SUVs). Bilosomes were prepared following the same procedure except for the presence of Na-DOC (Span 80: CH: Na-DOC in 2:1:0.1 mole ratio) in hydration medium along with TT. GM-bilosomes were prepared following the same procedure used for the preparation of plain bilosomes except inclusion of GM-OCM-DSPE (10% w/w of total lipid content) in the lipidic phase.

Physicochemical characterization

Size, PDI and zeta potential

The vesicle size and PDI of vesicular formulations were measured by dynamic light scattering (DLS) (Nano ZS, Malvern, UK) while zeta potential was estimated on the basis of electrophoretic mobility under an electric field.

Entrapment efficiency (EE)

Micro Bicinchoninic Acid (BCA) method for protein estimation was used to quantify entrapped TT in vesicular formulations. Unentrapped TT was separated by size exclusion chromatography using Sephadex G-75 column according to the method previously reported by our group.² Entrapped TT was liberated by disrupting the eluted vesicles (1 mL) using Triton X-100 (100 μ L, 1% v/v) and quantified using validated Micro BCA method at 561 nm.

Transmission electron microscopy

Surface morphology was examined using transmission electron microscopy (TEM, FEI, Technai G2 F20, FE TEM, USA) operated at 80 kV. Briefly, a drop of sample was placed over the formvar coated grid, stained with phosphotungstic acid (1% w/v) solution, air dried and subjected to TEM analysis.

Lyophilization

Developed vesicular formulations were freeze dried (Vir Tis, Wizard 2.0, USA) using freeze drying cycle comprised of (1) freezing at -60°C for 8 h, (2) primary drying at -60 to 20°C for 42 h and (3) secondary drying at 25°C for 2 h. The constant pressure of 200 torr was applied in each step. Different cryoprotectants viz. dextrose, sucrose, lactose, trehalose, mannitol, glycine and inulin at 5% w/v concentration were screened and lactose was finalized based on appearance of cake, redispersibility index and reconstitution score. Lactose was further optimized for different concentration of 2.5%–10% w/v.

Stability studies

In process stability of BSA

Chemical stability. Chemical integrity of entrapped TT into freeze dried formulations was examined using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). The entrapped TT from different vesicular formulations was extracted by disrupting vesicles with Triton X-100 (100 μL , 1% v/v) and loaded in the individual wells (stacking gel 5%, resolving gel 7.5%). Electrophoresis was carried out at 20 mA and 200 V followed by visualization of resolved bands by staining with Coomassie brilliant blue.

Conformation stability. Extracted TT samples (as in “Chemical stability” section) were further subjected to conformation stability using circular dichroism (CD, J-815, JASCO Apparatus, Japan). Extracted samples (~ 350 μL) were placed in rectangular quartz cuvette of path length 0.1 cm and analyzed in triplicate at 25°C using 1 nm bandwidth, in the wavelength range of 250–190 nm.²³

Stability in biological milieu

Vesicular formulations were incubated in simulated gastric fluid (SGF, pH 1.2) and simulated intestinal fluids (SIF, pH 6.8) and PBS (pH 7.4) having two different concentrations (5 mM and 20 mM) of Na-DOC. Vesicular formulations (200 μL) were diluted up to 10 mL with different media and incubated at 37°C for 2 h in SGF and 4 h in SIF and PBS having two different concentrations of Na-DOC. The overall effect on stability was evaluated by measuring the effect on size and percentage of TT retained within the vesicular formulations. The percentage of TT retained was calculated by considering the initial entrapped amount as 100%.

In-vitro APCs uptake

Cell culture

Murine macrophage cell line (RAW 264.7) was procured from National Centre for Cell Science (NCCS), Pune, India. RPMI-1640, supplemented with 10% fetal bovine serum and antibiotics was used as cell culture medium. Cells were grown at 37°C in a 5% CO_2 and 95% air humidified atmosphere and confluent cultures of cells were harvested by trypsinization. Cells were counted and suitably diluted to obtain 1×10^5 cells/well followed by overnight incubation for cell attachment.

Cellular uptake study

Cultured cells were incubated with coumarin-6 loaded vesicular formulations at different concentrations of 10, 30, 100 $\mu\text{g/mL}$ and incubated for 1, 3 and 6 h. Following incubation cells were thoroughly washed 6 times with PBS and visualized using confocal microscope [Model Olympus FV 1000]. For quantitative determination cells were lysed by adding 200 μL of 0.1% Triton X-100 and fluorescence was measured at 495 nm and 505 nm, excitation and emission wavelength, respectively.

Immunological studies

Immunization

Male BALB/c mice 6 weeks old procured from Central Animal Facility, NIPER, India, were used for immunization studies. Animals were housed in plastic cages having free access to food and water and kept under a 12 h light/dark cycles throughout the experimentation. All animal studies protocols were duly approved by Institutional Animal Ethics Committee (IAEC), NIPER, SAS Nagar, India. All the experiments were performed in accordance with the guidelines of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), India.

Animals were randomly divided into six groups (each containing 6 animals) and fasted before 3 h of oral administration. Mice were immunized orally with alum adsorbed TT, niosomes, bilosomes and GM-bilosomes in a dose equivalent to 5 Lf TT per mouse. Mice were also immunized intramuscularly with alum adsorbed TT (Adsorbed) I.P. (5 Lf/mice). Oral saline treated group was additionally taken as negative control. Secondary immunization was also done on day 22 in all the groups.

Sample collection

Blood samples were collected on day 0 (pre-immune), 7, 14, 21, 28 and 35 by retro-orbital puncture and serum was separated by centrifugation at 10,000 rpm for 8 min and stored at -20°C until analyzed for IgG titre using ELISA.²⁴

Saliva, intestinal fluids and fecal contents were collected on day 35 after intraperitoneal injection of pilocarpine (80 mg/kg) for the estimation of sIgA. After injection mice began to salivate within a couple of minutes, saliva was collected and pooled.^{24,25} Following saliva collection mice were sacrificed and intestinal sections of equal lengths were excised and stored in PBS (pH 7.4) at $2-8^{\circ}\text{C}$. The samples were vortexed and intestinal contents were settled by centrifugation at 20,000 rpm for 8 min, supernatant was analyzed for estimation of IgA titre using IgA ELISA kit. Fecal samples from each group were collected, pooled and processed similarly.

Endogenous levels of IL-2 and IFN- γ were estimated in spleen homogenate. Spleen was dissected and homogenized at 8000 rpm for 2 min using Ultra Turrex tissue homogenizer. Solid tissue contents were settled by centrifugation at 20,000 rpm for 8 min; supernatant was separated and analyzed for IL-2 and IFN- γ .²⁶

Measurement of antibody titre and cytokines levels

Commercially available ELISA kits were used to measure the antibody titres in serum and other biological fluids. Microtitre plates (Nunc Immuno™ Maxisorb F96 plates) were coated with

Table 1
Quality attributes of TT loaded vesicular formulations.

Formulation	Vesicle size (nm)	PDI	Zeta potential (mV)	EE (%)
Niosomes	156 ± 7	0.242 ± 0.028	+16.3 ± 1.4	77.2 ± 2.6
Bilosomes	147 ± 5	0.204 ± 0.035	−19.3 ± 3.1	73.6 ± 2.1
GM-bilosomes	164 ± 10	0.258 ± 0.041	−17.9 ± 2.8	75.8 ± 3.3

All values are represented as mean ± SD (n = 6).

100 µL of TT solution (1 Lf/mL) in PBS buffer followed by overnight incubation at 4 °C and washed thrice with PBS containing 0.05% of Tween 20 (PBST). After serially diluting the serum samples with PBS, 100 µL of the samples were added to the pre-coated plates and incubated at 37 °C for 2 h and washed thrice with PBST. IgG (γ-chain specific) peroxidase conjugate (100 µL) was added to each well, incubated at 37 °C for 1 h and washed with PBST. TMB substrate (100 µL) was added to the plates and incubated at room temperature for 30 min. H₂SO₄ (2 N, 100 µL) was added to stop the reaction and yellow color produced was measured spectrophotometrically at 450 nm.

sIgA was estimated following the same procedure except the use of IgA (α-chain specific) peroxidase conjugate instead of IgG (γ-chain specific) peroxidase conjugate used in IgG determination.

IL-2 and IFN-γ in spleen homogenates were determined using separate ELISA kits for individual cytokines following the protocol given by the manufacturer.

Statistical analysis

All *in-vitro* results have been expressed as the mean ± standard deviation (SD) while *in-vivo* results have been expressed as mean ± standard error mean (SEM). Statistical analysis was performed with Sigma Stat (Version 2.03) using one-way analysis of variance (ANOVA) followed by Tukey–Kramer multiple comparison test. $P < 0.05$ was considered as statistically significant.

Results

Preparation and optimization of vesicular formulations

The size, PDI, zeta potential and entrapment efficiency (EE) of different vesicular formulations are shown in Table 1. Morphology determination by TEM analysis (Figure 1) further confirmed the formation of vesicles of almost spherical shape with smooth surface. The results obtained in TEM analysis were also in good correlation with the results of DLS analysis.

Lyophilization

All vesicular formulations were lyophilized in order to impart long term stability. A number of cryoprotectants (Table 2) were screened and on the basis redispersibility index (Ri) lactose was finalized as it resulted in the formation of voluminous, easy to redisperse cake with Ri near one. However, a collapsed cake was observed in case of other cryoprotectants which resulted in either aggregation (high redispersibility index) and/or reversal of

surface charge of vesicular formulations after reconstitution. Based on the results of preliminary screening lactose was finalized and further optimized for different concentrations (2.5, 5.0, 7.5, 10% w/v). Lactose (5% w/v) resulted in redispersibility index close to 1 (Table 3). A significant increase ($P < 0.05$) in redispersibility index was observed at lower concentration (2.5% w/v) while the difference was insignificant ($P > 0.05$) at higher concentrations.

Stability studies

In process stability for BSA

Chemical stability. Chemical stability of the TT was maintained in all the vesicular formulations as evident by symmetrical position of bands (Figure 2, A). Moreover absence of additional bands in all the samples further confirmed the chemical stability of TT throughout the process.

Conformational stability. Overlay CD spectra of standard TT and TT extracted from different vesicular formulations are shown in Figure 2, B. TT was found conformationally stable throughout the process as evident by superimposed CD spectra of test samples with the CD spectrum of standard.

Stability studies in biological milieu

The effect of different biological media on size and % of TT retained, within the different formulations, is shown in Table 4. GM-bilosomes were found quite stable as evident by vesicle size and significantly ($P < 0.05$) higher percentage of TT retained in comparison with niosomes and bilosomes. Incubation in presence of Na-DOC solution (5 and 20 mM) resulted in significant loss ($P < 0.05$) of entrapped TT in case of niosomes as compared to bilosomes and GM-bilosomes.

In-vitro APCs uptake

Vesicular formulations exhibited higher uptake over free coumarin-6 as evident by higher green fluorescence observed in case of vesicular formulations in CLSM analysis (Figure 3, A). Quantitative determination by spectrofluorimetry revealed time and concentration dependent cellular uptake of vesicular formulations (Figure 3, B). At higher concentration (100 µg/mL), the uptake was increased by 1–2 folds after 1 h, 2–4 folds after 3 h and 6–12 folds after 6 h of incubations in comparison with free coumarin-6. GM-bilosomes exhibited significantly higher ($P < 0.001$) uptake in comparison with bilosomes and niosomes while this difference was insignificant ($P > 0.05$) between niosomes and bilosomes. GM-bilosomes exhibited 2.50 folds and 2.17 folds higher uptake in comparison with niosomes and bilosomes, respectively.

Immunological studies

End point titre method based on maximum dilution was used to measure serum antibody titre in which titers were expressed as the log₁₀ of the reciprocal of last dilution, which gave an optical density (OD) at 450 nm equivalent to the OD of negative control (control group of animals, which received only saline). The mean serum IgG level following

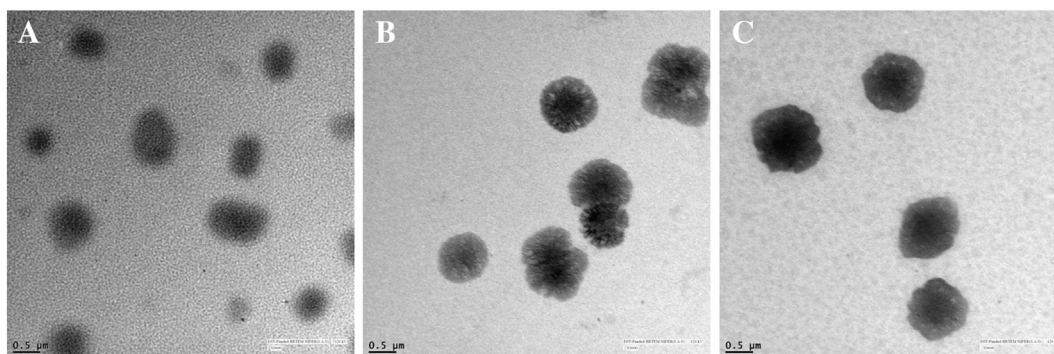


Figure 1. TEM images of TT loaded (A) Niosomes (B) Bilosomes and (C) GM-bilosomes.

Table 2

Redispersibility index and reconstitution score of various formulations after lyophilization using different cryoprotectants.

	Niosomes			Bilosomes			GM-bilosomes		
	Particle size (nm)	Ri = (Sf/Si)	RS	Particle size (nm)	Ri = (Sf/Si)	RS	Particle size (nm)	Ri = (Sf/Si)	RS
Initial	156 ± 7	–	–	147 ± 5	–	–	164 ± 10	–	–
Without cryoprotectant	ND	ND	ND	ND	ND	ND	ND	ND	ND
Dextrose	264 ± 17	1.69 ± 0.11	**	198 ± 17	1.35 ± 0.12	**	187 ± 10	1.14 ± 0.06	**
Sucrose	231 ± 18	1.48 ± 0.12	***	164 ± 10	1.12 ± 0.06	***	174 ± 12	1.06 ± 0.07	***
Lactose	177 ± 9	1.13 ± 0.05	***	150 ± 5	1.02 ± 0.03	***	166 ± 5	1.01 ± 0.03	***
Trehalose	255 ± 12	1.63 ± 0.08	**	181 ± 8	1.23 ± 0.05	**	200 ± 7	1.22 ± 0.04	**
Mannitol	ND	ND	ND	ND	ND	ND	ND	ND	ND
Glycine	ND	ND	ND	ND	ND	ND	ND	ND	ND
Inulin	ND	ND	ND	ND	ND	ND	ND	ND	ND

ND: not determined as non-redispersed collapsed cake was formed; RS: Reconstitution score (*** redispersible within 20 s with mere shaking, ** redispersible within 1 min with high shear vortexing).

administration of different formulations is shown in Figure 4. At the end of the day 35, orally administered free TT elicited the lowest IgG titre among all the formulations. A significantly higher ($P < 0.01$) IgG titre was observed in case of niosomes as compared to orally administered free TT. Bilosomes further elicited significantly higher titre in comparison with niosomes ($P < 0.05$) and orally administered free TT ($P < 0.001$). In line, GM-bilosomes further improved the IgG titre which was significantly higher in comparison with oral TT ($P < 0.001$), niosomes ($P < 0.001$) and bilosomes ($P < 0.05$). TT administered through i.m. route resulted in the highest IgG titre. Following primary dosing slight increase in immune response was observed within the span of 7–14 days followed by slight decrease in immune response within the span of 14–21 days. Subsequently, all animal groups were treated with booster dose at day 21 following which a significant improvement in immune response was observed at the end of the day 35. At the end of the day 35, GM-bilosomes exhibited 2.0 and 1.4 folds higher immune response in comparison with niosomes and bilosomes, respectively.

Mucosal immune response measured as sIgA level in saliva, intestinal secretions and fecal contents is shown in Figure 5, A, B and C, respectively. A very low sIgA titre in all the secretions was observed in case of free TT administered through oral and i.m. routes while it was quite higher in case of different vesicular formulations. Interestingly, the trend was quite similar with our observation of serum IgG level as GM-bilosomes > bilosomes > niosomes.

Endogenous cytokines levels of IL-2 and IFN- γ estimated in spleen homogenate at the day 35 are shown in Figure 6, A and B, respectively. Significantly higher ($P < 0.05$) levels of both IL-2 and IFN- γ were observed in case of bilosomes as compared to control, free TT administered by i.m. and oral routes and niosomes. GM-bilosomes further fortified the immune response as a significantly higher level of both cytokines was observed in comparison with control, free TT administered by i.m. and oral routes, niosomes ($P < 0.001$) and plain bilosomes ($P < 0.05$).

Discussion

In the present report mannosylated bilosomes were proposed for selective uptake and delivery of TT to APCs through the oral route. GM has been reported to exhibit excellent stability against digestive enzymes^{19,20} which can also increase the stability of bilosomes in GIT. Furthermore, polymeric nature can provide higher density of mannose molecules over the bilosomes surface which can result in more precise recognition and binding to mannose receptors over expressed on APCs. Therefore, we hypothesized that dual specification, of higher stability against gastric enzymes and selective and enhanced uptake through APCs, can ultimately improve the performance of proposed GM-bilosomes, in terms of eliciting better immune response. For the purpose GM-OCM-DSPE conjugate was chemically synthesized and authenticated by FTIR and NMR analysis (Overlay spectra

Table 3

Redispersibility index and reconstitution score of different formulations after lyophilization with different concentration of lactose.

	Niosomes			Bilosomes			GM-bilosomes		
	Particle size (nm)	Ri = (Sf/Si)	RS	Particle size (nm)	Ri = (Sf/Si)	RS	Particle size (nm)	Ri = (Sf/Si)	RS
Initial	156 ± 7	—	—	147 ± 5	—	—	164 ± 10	—	—
2.5%	209 ± 12	1.34 ± 0.07	**	175 ± 14	1.19 ± 0.09	**	191 ± 11	1.16 ± 0.11	**
5%	177 ± 9	1.13 ± 0.05	***	150 ± 5	1.02 ± 0.03	***	166 ± 5	1.01 ± 0.03	***
7.5%	204 ± 16	1.31 ± 0.10	***	172 ± 14	1.17 ± 0.09	***	180 ± 8	1.10 ± 0.05	***
10%	239 ± 13	1.35 ± 0.07	***	191 ± 15	1.30 ± 0.10	***	199 ± 10	1.22 ± 0.06	***

RS: Reconstitution score (*** redispersible within 20 s with mere mixing, ** redispersible within 1 min with high shear vortexing).

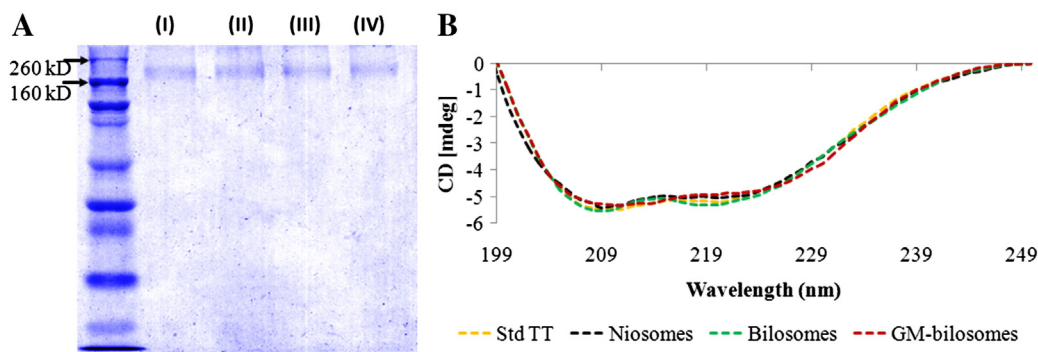


Figure 2. Stability studies. (A) Chemical stability where lanes I to IV represent resolved bands obtained in SDS-PAGE for standard TT and TT extracted from Niosomes, Bilosomes and GM-bilosomes respectively. (B) Conformation stability where overlay CD spectra represent standard TT and TT extracted from different formulations.

with peak assignment and sufficient details have been provided in the supplementary material). Matrix-assisted laser ionization/desorption–time of flight (MALDI TOF TOF, Bruker) further revealed 12%–19% degree of substitution of GM in final conjugate (Detailed information has been provided in the supplementary material). Selection of formulation components at definite weight ratio and other processing variables was based on our previous experience in similar direction.^{1,2,13}

In order to impart the long term storage stability vesicular formulations were freeze dried²⁷ and lactose was finalized in preliminary screening based on formation of voluminous cake with excellent redispersibility index. A clear aggregation was observed in case of mannitol, glycine and inulin which could be attributed to the formation of ice crystals in the case of mannitol and inulin, and adsorption and charge reversal in the case of inulin. Although the difference in redispersibility index in case of dextrose, sucrose and trehalose was insignificant ($P > 0.05$) yet, eliminated from consideration based on cake appearance and reconstitution score. Further, lactose resulted in formation of voluminous, easy to redisperse cake at all tested concentrations. High redispersibility index observed at lower concentration (2.5% w/v) could be attributed to the insufficient amount of cryoprotectant ultimately required to protect the vesicles against the stress of freeze drying. Insignificant change in quality attributes was observed at higher concentration of cryoprotectant therefore 5% w/v lactose was optimized for freeze drying of vesicular formulations.

Chemical stability of the TT entrapped within different freeze dried vesicular formulations was confirmed by SDS-PAGE indicating the suitability of the method and formulation for delivery of antigens. Furthermore, superimposed spectra of

standard and test TT samples and absorption bands between 190 and 250 nm, characteristic of α -helicity of secondary structure²³ further supported the appropriateness of the method and formulation for delivery of antigens. Stability of vesicular formulations in different bio-mimetic media was performed in order to establish the protective potential of the developed system. Higher stability against bile salts dependent surface solubilization exhibited by bilosomes over niosomes could be attributed to repulsion between the bile salts already present in the vesicle bilayer and the external bile salts in the solution.²⁸ Further enhanced stability exhibited by GM-bilosomes, in terms of percentage of TT retained within the system, might be the consequence of additional polymeric coating barrier.

In-vitro release studies were also performed in PBS and selection of PBS as release medium was based on its simulating nature with other biological fluids.^{29–31} It is very well documented that nanocarriers are absorbed by specialized transport system via membranous epithelial cells (M-cells) of the Peyer's patches present along the gastrointestinal tract. After their rapid uptake by M-cells they enter into the systemic circulation via lymphatic drainage and slowly release the drug for a longer period of time. Therefore, *in-vitro* drug release was carried out as a quality control tool at physiological pH (7.4). Sustained release observed in case of GM-bilosomes might be the consequence of polymeric coating barrier of GM (Figure 4S, supplementary material).

Macrophages are the APCs present in the vicinity of Peyer's patches.³² Cell uptake study was performed in order to mimic and determine the macrophage uptake selectivity of vesicular formulations using RAW 264.7 cell lines. Selection of RAW 264.7 macrophage cell line was based on its universal acceptability to evaluate uptake of vaccine adjuvants. Coumarin-6

Table 4

Effect of simulated biological milieu on quality attributes of different vesicular formulations.

Biological milieu	Niosomes		Bilosomes		GM-bilosomes	
	Size (nm)	% TT retained	Size (nm)	% TT retained	Size (nm)	% TT retained
Initial	156 ± 7	100	147 ± 5	100	164 ± 10	100
SGF (pH 1.2)	173 ± 9	72.1 ± 2.3	159 ± 5	75.3 ± 2.2	174 ± 8	84.4 ± 3.1
SIF (pH 6.8)	160 ± 8	65.6 ± 1.4	153 ± 7	68.9 ± 3.2	169 ± 7	78.3 ± 2.2
5 mM Na-DOC solution	192 ± 10	58.1 ± 2.7	154 ± 6	71.6 ± 2.7	170 ± 5	77.3 ± 2.1
20 mM Na-DOC solution	436 ± 42	43.6 ± 5.3	158 ± 5	66.1 ± 3.4	175 ± 8	73.5 ± 3.0

All values are represented as mean ± SD, (n = 6).

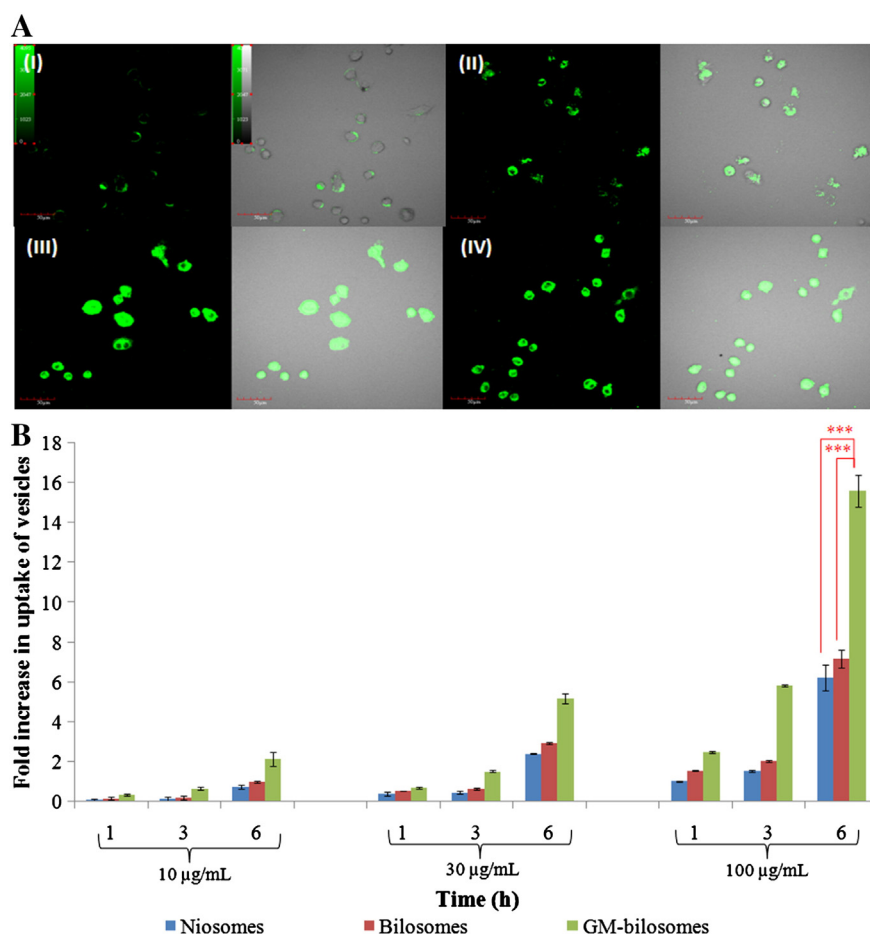


Figure 3. (A) CLSM image (10×) showing macrophage uptake of (I) free coumarin-6; (II) Niosomes; (III) Bilosomes; and (IV) GM-bilosomes. For each incubation type, the left and right panels represent green fluorescence and overly of green fluorescence and differential interference contrast image. (B) Quantitative determination of macrophage uptake of coumarin-6 loaded vesicular formulations. (***) $P < 0.001$.

loaded vesicular formulations exhibited significantly higher uptake in comparison to free coumarin-6. This observation is in agreement with previous reports in which vesicular nature and negative surface have been reported as important factors which play a major role in cellular uptake by the scavenger receptors.^{33,34} The significantly higher uptake observed in the case of GM-bilosomes could be attributed to more precise recognition and selective uptake of GM-bilosomes by mannose receptors abundantly present on the macrophages.³⁵

Higher macrophage uptake and subsequent exposure of antigen to the antigen presenting cells (APCs) can induce T-cell

mediated immunity which can ultimately fortify the immune response.³⁶ Minimal IgG titre observed in case of free TT could be ascribed to the obvious reason of degradation of free antigen in harsh GI environment. Improved performance observed in case of niosomes as compared to free TT could be attributed to some degree of protection provided by vesicular structure to the entrapped TT against external environment. This observation is in agreement with our previous findings.^{1,2} Inclusion of bile salts into vesicles further improved the performance as evident by the higher IgG titre observed in case of bilosomes. This observation further supported the results of *in-vitro* stability study in

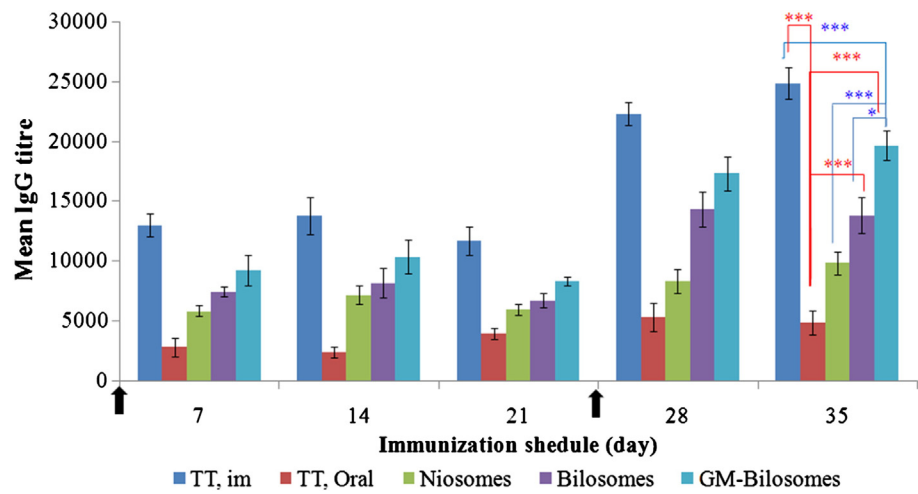


Figure 4. Serum IgG titre following administration of TT (oral and i.m.) and TT loaded niosomes, bilosomes and GM-bilosomes. Arrow (↑) represents the day of dosing. Data are presented as mean ± SEM (n = 6) (***P* < 0.001, **P* < 0.05).

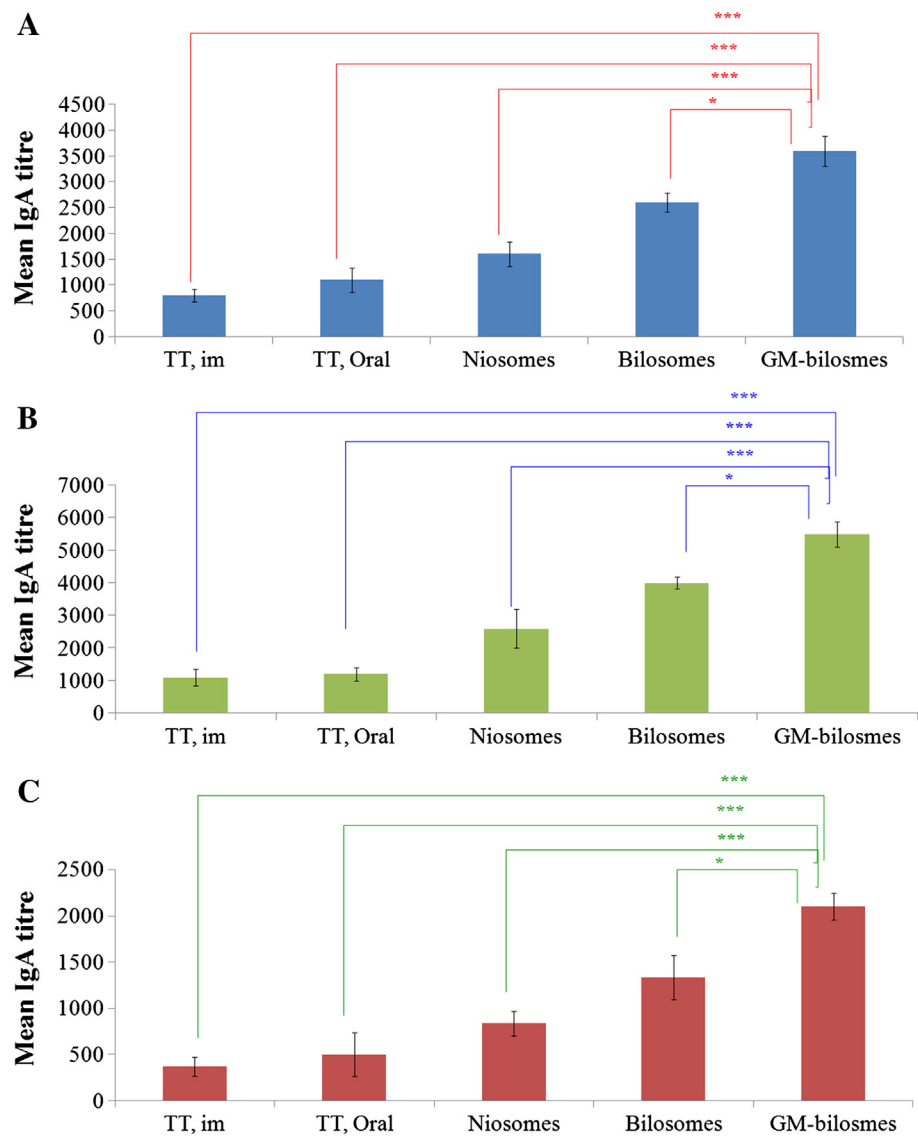


Figure 5. sIgA titre in (A) saliva (B) intestinal fluid and (C) fecal content following administration of free TT (oral and i.m.) and TT loaded niosomes, bilosomes and GM-bilosomes. Data are presented as mean ± SEM (n = 6) (***P* < 0.001, **P* < 0.05).

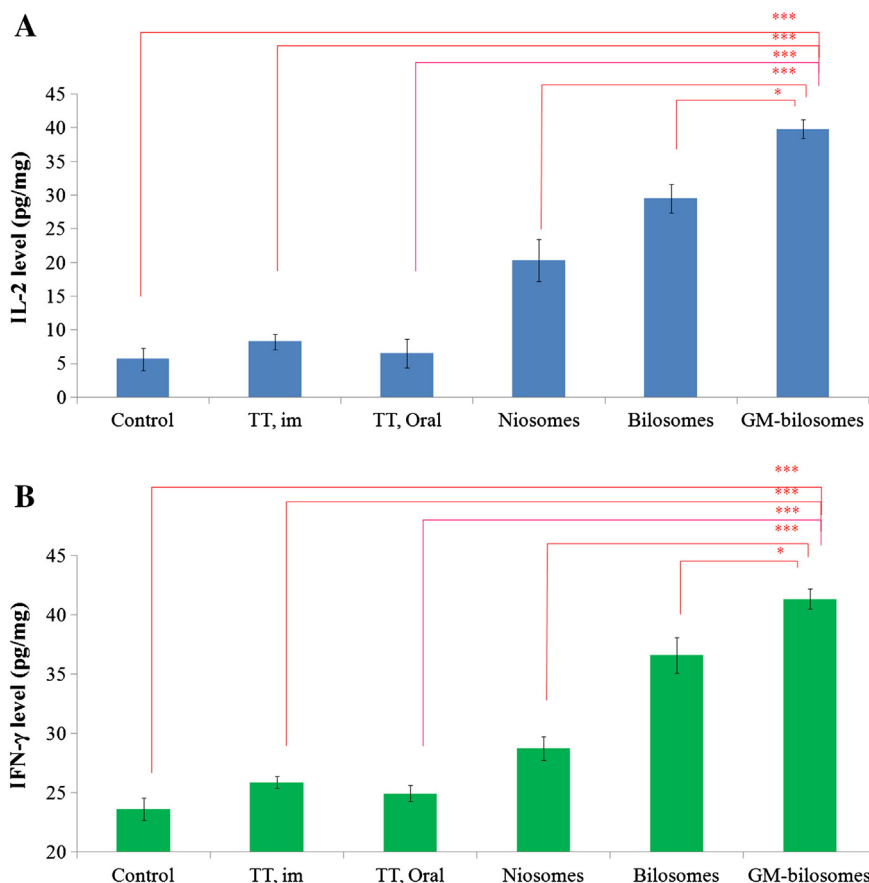


Figure 6. Cytokines level in spleen homogenate following administration of TT (oral and i.m.) and TT loaded niosomes, bilosomes and GM-bilosomes **(A)** IL-2 **(B)** IFN- γ . Data are presented as mean $\pm$ SEM ($n = 6$) (** $P < 0.001$, * $P < 0.05$).

biomimetic media in which bilosomes exhibited improved stability over niosomes. This observation is also in coordination with our previous findings in which bilosomes were found superior in comparison with niosomes.¹³ Mannosylation further improved the performance which might be the consequence of additional stability against gastric enzymes and recognition and selective uptake of GM-bilosomes by macrophages and dendritic cells (the major APCs present in the vicinity of Peyer's patches). Furthermore, intrinsic immunogenic effect of mannose might have fortified the immunogenic response.¹ Highest immunogenic response observed in case of i.m. TT could be attributed to the adjuvanted TT and sustained release because of depot formation at the site of injection.³⁵ Similarly GM-bilosomes elicited significantly higher sIgA level in comparison with niosomes and plain bilosomes which could be attributed to the same reason of protection and selective uptake and presentation to APCs present across the mucosal linings. In contrast to IgG, i.m. TT produced negligible sIgA because of the inability to stimulate mucosal immune system. The results are in agreement with our previous findings.^{1,2} Therefore, oral route can also present additional advantage of mucosal immunity i.e. neutralization of antigens at their first exposure to mucosal linings. Conventional immunization generates strong antibody response since antigens are presented by B cells through MHC class II pathway to Th2 cells. Besides this pathway, oral immunization also results in Th1/cell mediated

immune response through MHC class I pathway which fortifies the immune response by providing therapeutic immunization.¹ Generation of cell mediated response can be identified by higher level of IL-2 and IFN- γ which are Th1 dependent cytokines. Significantly lower cytokines levels in case of i.m. TT confirmed the inability of conventional parenteral route to elicit cell mediated immune response and its restricted use for the purpose of prophylactic immunization only. The findings are in harmonization with previous reports in which conventional immunization has been reported incompetent to elicit cellular response.^{1,37} Significantly higher level of cytokines produced in case of GM-bilosomes revealed its potential in both prophylactic and therapeutic immunization. Conclusively GM-bilosomes were found to elicit good systemic, mucosal as well as cellular immune response and can be considered as a promising carrier and adjuvant for non-invasive oral immunization. Besides the performance in eliciting good immune response simplicity, economical approach, patient compliance and technical feasibility to scale up are other hidden advantages which strongly support the candidature of the developed formulation for mass immunization. Cumulatively, these findings strengthen our expectation that GM-bilosomes can be exploited as a potential alternative to parenteral immunization. These findings, in turn are expected to benefit the design and development of vesicular carriers for variety of antigens other than TT.

Acknowledgment

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <http://dx.doi.org/10.1016/j.nano.2013.08.012>.

References

- Jain S, Singh P, Mishra V, Vyas SP. Mannosylated niosomes as adjuvant-carrier system for oral genetic immunization against hepatitis B. *Immunol Lett* 2005;**101**:41–9.
- Jain S, Vyas SP. Mannosylated niosomes as adjuvant-carrier system for oral mucosal immunization. *J Liposome Res* 2006;**16**:331–45.
- Rothkotter HJ, Pabst R, Bailey M. Lymphocyte migration in the intestinal mucosa: entry, transit and emigration of lymphoid cells and the influence of antigen. *Vet Immunol Immunopathol* 1999;**72**:157–65.
- Des Rieux A, Fievez V, Garinot M, Schneider YJ, Preat V. Nanoparticles as potential oral delivery systems of proteins and vaccines: a mechanistic approach. *J Control Release* 2006;**116**:1–27.
- Neutra MR, Kozlowski PA. Mucosal vaccines: the promise and the challenge. *Nat Rev Immunol* 2006;**6**:148–58.
- Giddam AK, Zaman M, Skwarczynski M, Toth I. Liposome-based delivery system for vaccine candidates: constructing an effective formulation. *Nanomedicine* 2012;**7**:1877–93.
- Uchegbu IF, Vyas SP. Non-ionic surfactant based vesicles (niosomes) in drug delivery. *Int J Pharm* 1998;**172**:33–70.
- van der Lubben IM, Verhoef JC, van Aelst AC, Borchard G, Junginger HE. Chitosan microparticles for oral vaccination: preparation, characterization and preliminary in vivo uptake studies in murine Peyer's patches. *Biomaterials* 2001;**22**:687–94.
- Borges O, Borchard G, Verhoef JC, de Sousa A, Junginger HE. Preparation of coated nanoparticles for a new mucosal vaccine delivery system. *Int J Pharm* 2005;**299**:155–66.
- Mann JF, Scales HE, Shakir E, Alexander J, Carter KC, Mullen AB, et al. Oral delivery of tetanus toxoid using vesicles containing bile salts (bilosomes) induces significant systemic and mucosal immunity. *Methods* 2006;**38**:90–5.
- Myschik J, Rades T, Hook S. Advances in lipid-based subunit vaccine formulations. *Curr Immunol Rev* 2009;**5**:42–8.
- Romero EL, Morilla MJ. Topical and mucosal liposomes for vaccine delivery. *Wiley Interdiscip Rev Nanomed Nanobiotechnol* 2011;**3**:356–75.
- Singh P, Prabakaran D, Jain S, Mishra V, Jaganathan KS, Vyas SP. Cholera toxin B subunit conjugated bile salt stabilized vesicles (bilosomes) for oral immunization. *Int J Pharm* 2004;**278**:379–90.
- Frey A, Giannasca KT, Weltzin R, Giannasca PJ, Reggio H, Lencer WI, et al. Role of the glycocalyx in regulating access of microparticles to apical plasma membranes of intestinal epithelial cells: implications for microbial attachment and oral vaccine targeting. *J Exp Med* 1996;**184**:1045–59.
- Jani P, Halbert GW, Langridge J, Florence AT. The uptake and translocation of latex nanospheres and microspheres after oral administration to rats. *J Pharm Pharmacol* 1989;**41**:809–12.
- Ahsan F, Rivas IP, Khan MA, Torres Suarez AI. Targeting to macrophages: role of physicochemical properties of particulate carriers—liposomes and microspheres—on the phagocytosis by macrophages. *J Control Release* 2002;**79**:29–40.
- Cuna M, Alonso-Sandel M, Remunan-Lopez C, Pivel JP, Alonso-Lebrero JL, Alonso MJ. Development of phosphorylated glucomannan-coated chitosan nanoparticles as nanocarriers for protein delivery. *J Nanosci Nanotechnol* 2006;**6**:2887–95.
- Alonso-Sande M, Cuña M, Remuñán-López C, Teijeiro-Osorio D, Alonso-Lebrero JL, Alonso MJ. Formation of new glucomannan-chitosan nanoparticles and study of their ability to associate and deliver proteins. *Macromolecules* 2006;**39**:4152–8.
- Wang K, He Z. Alginate-konjac glucomannan-chitosan beads as controlled release matrix. *Int J Pharm* 2002;**244**:117–26.
- Du J, Sun R, Zhang S, Zhang LF, Xiong CD, Peng YX. Novel polyelectrolyte carboxymethyl konjac glucomannan-chitosan nanoparticles for drug delivery. I. Physicochemical characterization of the carboxymethyl konjac glucomannan-chitosan nanoparticles. *Biopolymers* 2005;**78**:1–8.
- Agrawal AK, Das M, Jain S. In situ gel systems as 'smart' carriers for sustained ocular drug delivery. *Expert Opin Drug Deliv* 2012;**9**:383–402.
- An NT, Dong NT, Dung PL, Thien DT. Synthesis and characterization of water-soluble O-carboxymethyl glucomannan derivatives. *Carbohydr Polym* 2011;**83**:645–52.
- Jain S, Rathi VV, Jain AK, Das M, Godugu C. Folate-decorated PLGA nanoparticles as a rationally designed vehicle for the oral delivery of insulin. *Nanomedicine (Lond)* 2012;**7**:1311–37.
- Sarti F, Perera G, Hintzen F, Kotti K, Karageorgiou V, Kammona O, et al. In vivo evidence of oral vaccination with PLGA nanoparticles containing the immunostimulant monophosphoryl lipid A. *Biomaterials* 2011;**32**:4052–7.
- Vila A, Sanchez A, Janes K, Behrens I, Kissel T, Vila Jato JL, et al. Low molecular weight chitosan nanoparticles as new carriers for nasal vaccine delivery in mice. *Eur J Pharm Biopharm* 2004;**57**:123–31.
- Zhu Y, Li X, Chen C, Wang F, Li J, Hu C, et al. Effects of aluminum trichloride on the trace elements and cytokines in the spleen of rats. *Food Chem Toxicol* 2012;**50**:2911–5.
- Jain S, Kumar D, Swarnakar NK, Thanki K. Polyelectrolyte stabilized multilayered liposomes for oral delivery of paclitaxel. *Biomaterials* 2012;**33**:6758–68.
- Conacher M, Alexander J, Brewer JM. Oral immunisation with peptide and protein antigens by formulation in lipid vesicles incorporating bile salts (bilosomes). *Vaccine* 2001;**19**:2965–74.
- Agrawal A, Gupta P, Khanna A, Sharma R, Chandrabanshi H, Gupta N, et al. Development and characterization of in situ gel system for nasal insulin delivery. *Pharmazie* 2010;**65**:188–93.
- Jain S, Patil SR, Swarnakar NK, Agrawal AK. Oral delivery of doxorubicin using novel polyelectrolyte-stabilized liposomes (layerosomes). *Mol Pharmaceutics* 2012;**9**:2626–35.
- Choudhary H, Agrawal A, Malviya R, Yadav S, Jaliwala Y, Patil U. Evaluation and optimization of preparative variables for controlled-release floating microspheres of levodopa/carbidopa. *Pharmazie* 2010;**65**:194–8.
- Hopkins S, Niedergang F, Corthesy-Theulaz I, Kraehenbuhl J. A recombinant Salmonella typhimurium vaccine strain is taken up and survives within murine Peyer's patch dendritic cells. *Cell Microbiol* 2000;**2**:59–68.
- Furumoto K, Nagayama S, Ogawara K, Takakura Y, Hashida M, Higaki K, et al. Hepatic uptake of negatively charged particles in rats: possible involvement of serum proteins in recognition by scavenger receptor. *J Control Release* 2004;**97**:133–41.
- Jain S, Kumar S, Agrawal AK, Thanki K, Banerjee UC. Enhanced transfection efficiency and reduced cytotoxicity of novel lipid-polymer hybrid nanoplexes. *Mol Pharmaceutics* 2013;**10**:2416–25.
- Tacken PJ, Fidor CG. Targeted antigen delivery and activation of dendritic cells in vivo: steps towards cost effective vaccines. *Semin Immunol* 2011;**23**:12–20.
- Mackay IR, Rosen FS, Ada G. Vaccines and vaccination. *N Engl J Med* 2001;**345**:1042–53.
- Belardelli F. Role of interferons and other cytokines in the regulation of the immune response. *APMIS* 2009;**103**:161–79.

**Improved stability and immunological potential of tetanus toxoid containing surface
engineered bilosomes following oral administration**

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1. Synthesis of Glucomannan-o-Carboxymethyl-Distearylphosphatidyl ethanolamine (GM-OCM-DSPE) conjugate

The Synthesis of GM-OCM-DSPE conjugate was carried out in two steps as shown in scheme (Figure 1S).

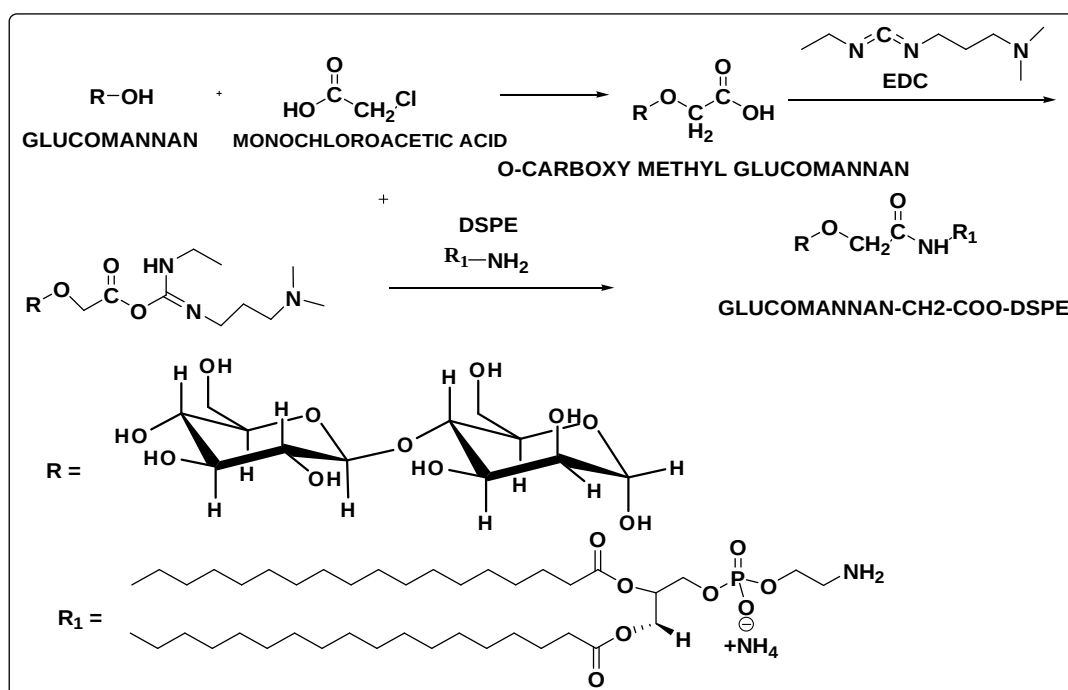


Figure 1S: Schematic presentation of the synthesis of GM-OCM-DSPE

In the first step, GM was converted to intermediate, o-carboxy methylated glucomannan (GM-OCM) by already reported method. Briefly, 250 mg of GM and 1 g of monochloroacetic acid were added in 100 mL of water by vigorous stirring for 30 min and pH was adjusted to approximately 10 by using 10% NaOH solution. The resultant solution was maintained at 60°C for 3 h and filtered. This filtrate was cooled to ambient temperature and pH was adjusted to approximately 5 using 1N HCl solution to precipitate out the GM-OCM which was filtered off and washed with 90% ethanol. This GM-OCM was further vacuum dried at 50°C and subsequently activated using EDC. Activation was done by dissolving GM-OCM and EDC in water and keeping this reaction mixture for overnight stirring. Subsequently, 200 mg of DSPE in tetrahydrofuran was added to above reaction mixture and kept for overnight stirring which finally resulted in formation of GM-OCM-DSPE conjugate. The synthesis of intermediate and

final conjugate was authenticated via Fourier transform infrared (FTIR) and nuclear magnetic resonance (NMR) spectroscopy. The FTIR spectrum of GM, GM-OCM and GM-OCM-DSPE were obtained on FTIR spectrometer (Nicolet Impact 410, Perkin Elmer Instrument, MA, USA) over the range 400-4000 cm^{-1} . Dry KBr (50 mg) was finely ground in mortar and samples (1-2 mg) were subsequently added and gently mixed. A manual press was used to form the pellets. For NMR analysis, GM and GM-OCM were dissolved in D_2O while GM-OCM-DSPE was dissolved in DMSO-d_6 . ^1H -NMR analyses were carried out in a Bruker DRX 400 MHz NMR spectrometer (Billerica, MA, USA).

1.1. FTIR analysis

The Comparative FTIR spectra of GM, GM-OCM and synthesized conjugate (GM-OCM-DSPE) is shown in Figure 2S.

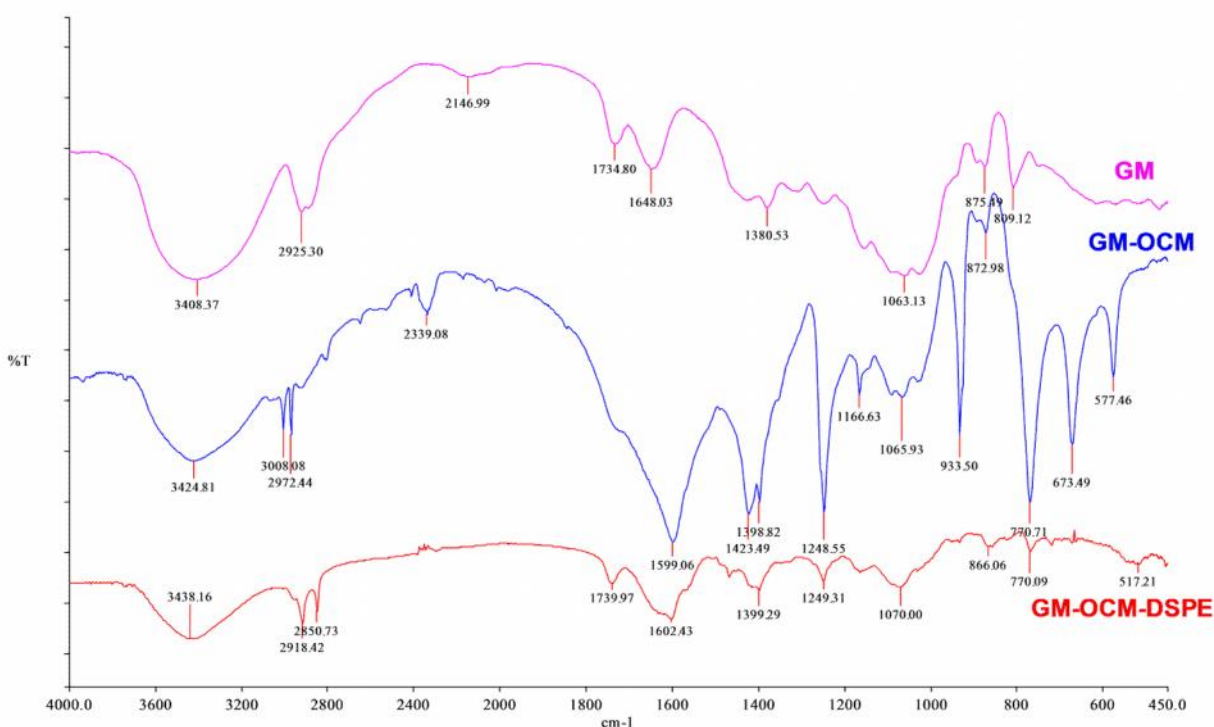


Figure 2S: Comparative FT-IR spectra for GM, GM-OCM and GM-OCM-DSPE

The FTIR spectrum of GM showed a characteristic peak at 1734 cm^{-1} which could be attributed to C=O stretching of the free acetyl groups present in the GM, while GM-OCM demonstrated broad shoulder in the range of $1750\text{--}1600\text{ cm}^{-1}$ which could be attributed to superimposition between free carboxyl groups of the GM-OCM and free acetyl groups native to the structure of

GM. The blue shift may be ascribed to intra and inter molecular H-bonding between free carboxyl and hydroxyl groups of the carboxymethylated sugar. Characteristic peaks at 1739 and 2850 cm^{-1} were observed for synthesized conjugate (GM-OCM-DSPE). The reappearance of the peak at 1739 cm^{-1} in the final conjugate (GM-OCM-DSPE) could be attributed to the formation of amide bond between free carboxyl group of GM-OCM and amine terminated DSPE. Also, the new peak observed in GM-OCM-DSPE at 2850 cm^{-1} could be attributed to $-\text{C}-\text{H}$ stretching frequency contributed by the $-\text{CH}_2$ linkage of DSPE in the final product.

1.2. NMR analysis

The overlay ^1H NMR spectra of GM, GM-OCM and GM-OCM-DSPE is shown in Figure 3S while individual spectrum with signal interaction are shown in Figure 4S, 5S and 6S, respectively.

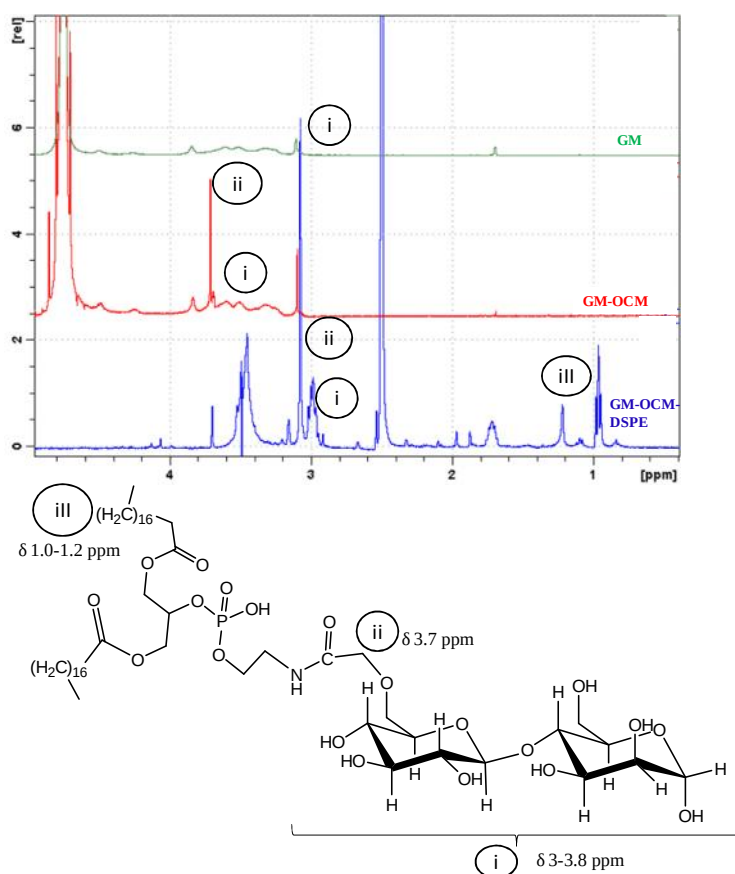


Figure 3S: NMR spectrum of (A) GM (B) GM-OCM and (C) GM-OCM-DSPE

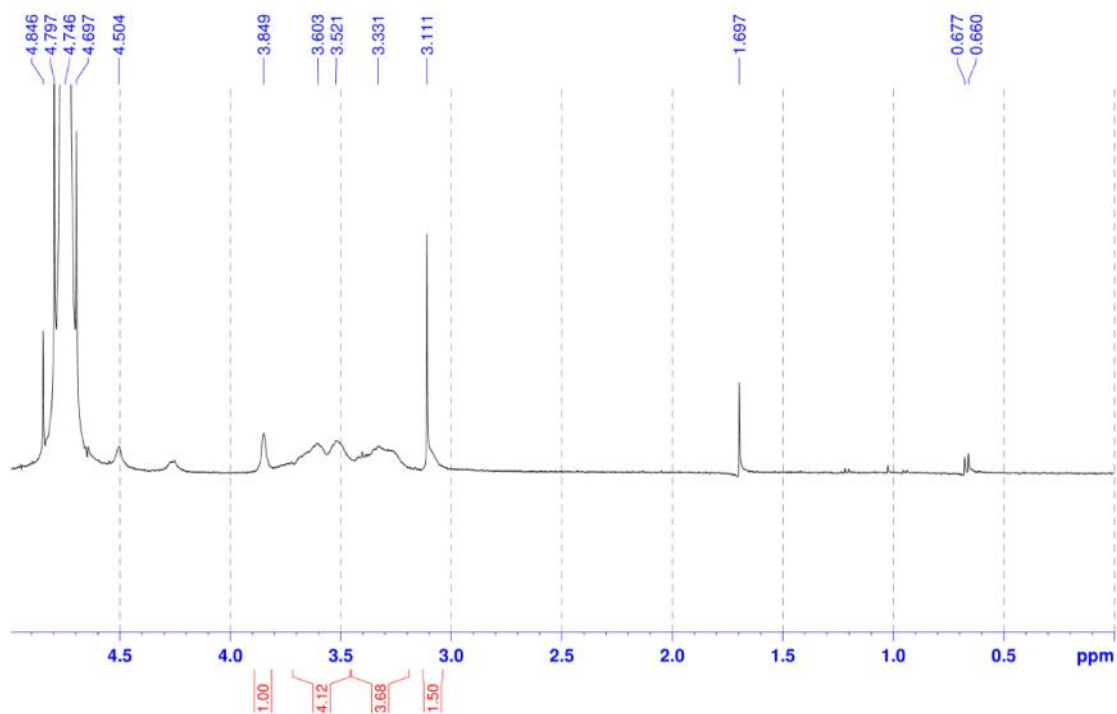


Figure 4S: Proton spectra of GM

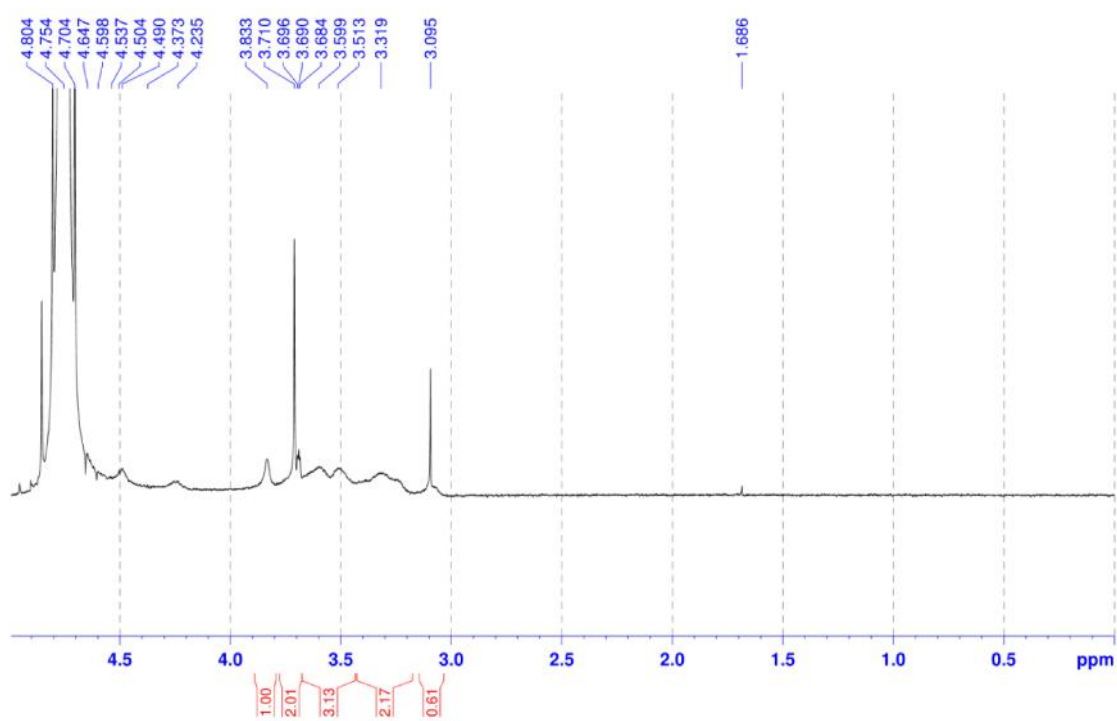


Figure 5S: Proton spectra of GM-OCM

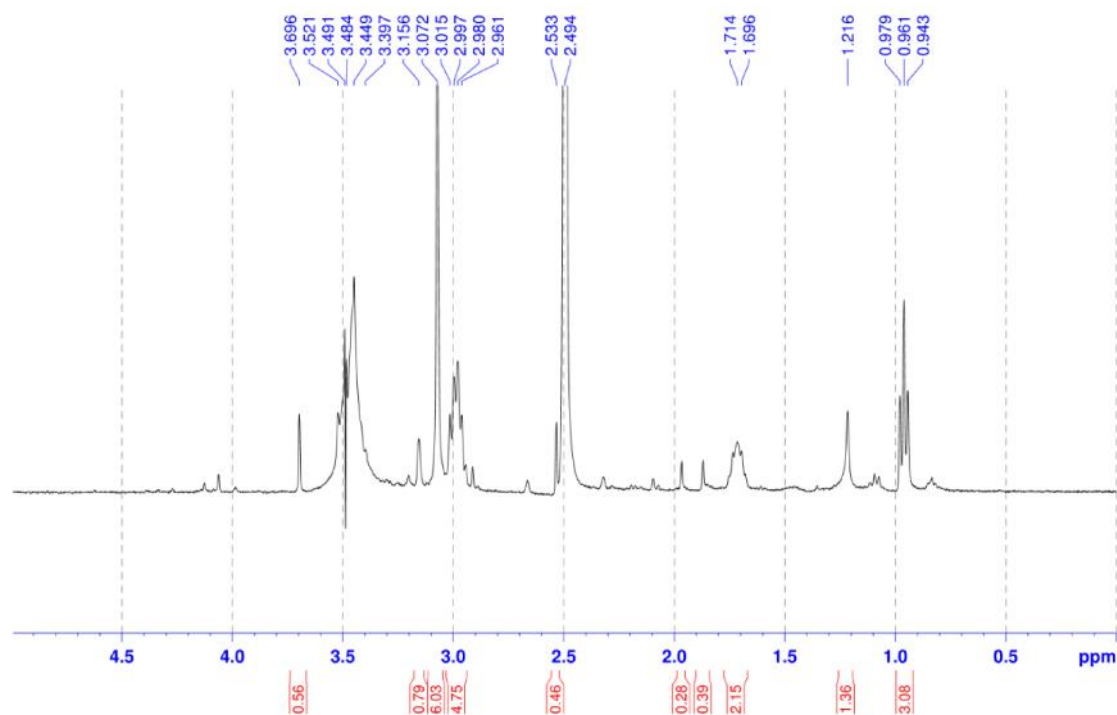


Figure 6S: Proton spectra of GM-OCM-DSPE

The proton peaks at δ 3-3.8 ppm were observed in GM, while new proton peak appeared in GM-OCM at δ 3.7 ppm. Additional peaks were observed at δ 2.9-3.5 ppm and δ 1-1.2 ppm in the final synthesized conjugate (GM-OCM-DSPE). The appearance of complex peaks at δ 3-3.8 ppm in GM could be attributed to numerous H1, H2, H3, H4, H5 and H6 of monosaccharide units (glucose and mannose). The appearance of new peak at δ 3.7 ppm in GM-OCM could be attributed to the $-\text{CH}_2$ group inserted due to substitution with monochloroacetic acid. A prominent peak at δ 4.7 ppm could be attributed to solvent D_2O (duteriated water). A peak at δ $\sim$ 3.1 ppm was observed in the spectrum of both GM and GM-OCM which could be attributed to $-\text{CH}_2(\text{C6})$ group of the monosaccharide side chain participating in bond formation with monochloroacetic acid. Conversion of GM-OCM to GM-OCM-DSPE was confirmed by shifting of δ 3-3.8 ppm to δ 2.9-3.5 ppm, which was appeared due to the protons attached to C1-C6 carbon of the monosaccharide units. It could be attributed to change in proton environment of GM due to steric hindrance of bulky DSPE. The peaks at around δ 1-1.2 ppm could be attributed to characteristic stearic acid ($-\text{C}_{17}\text{H}_{35}$) chain of DSPE. A sharp peak at δ 2.49 ppm could be attributed to the DMSO-d (duteriated dimethyl sulphoxide) which was used to solubilize GM-OCM-DSPE.

1.3. Matrix-assisted laser ionization/desorption (MALDI)

Matrix-assisted laser ionization/desorption – time of flight (MALDI TOF TOF, Bruker) was performed to determine the molar mass distribution of the components, intermediate and final conjugate. The tandem mass spectra of GM, GM-OCM and GM-OCM-DSPE are shown in Figure 7S-9S. However, polymers with high polydispersity (molecular weight of GM varies in the range of 150-200 kD) are very difficult to characterize with MALDI as signal intensity refinement is very poor against higher mass oligomers. The information obtained by MALDI was used to determine the degree of substitution in different conjugates (Table 1). Spectral analysis clearly indicated that mono-acetylation (>125%) was impossible as units were increased from 527 to 656 (if highest first base peak (m/z 94776) was considered) which ruled out the possibility of mono-acetylation and compound was supposed to undergo di-acetylation. The amount of di-acetylation was ~100%. However, such mono-acetylation was ~80% and di-acetylation was ~66%, if “second base peak (m/z = 146187)” was considered which can be considered the highest possible way of substitution. Similarly, 35% mono-DSPEylation and 19% Di-DSPEylation of the final conjugate was observed if “first base peak (m/z = 94776)” was taken into consideration, while it was 23 % and 12% if “second base peak” was considered. The results suggest the degree of substitution in the range of 12 to 19% if we consider di-acetylation as most possible route and suggest that almost 5 to 10 monosaccharide units were required to incorporate single DSPE molecule.

Table 1: Parameters evaluated using MALDI to determine the percentage of substitution

Parameters	GM-OCM		GM-OCM-DSPE	
	94776	146187	94776	146187
Mono-acetylation	125*	81	-	-
Di-acetylation	101	66	-	-
Mono-DSPEylation	-	-	35*	23
Di-DSPEylation	-	-	19	12

*ruled out as substitution greater than parent group was impossible

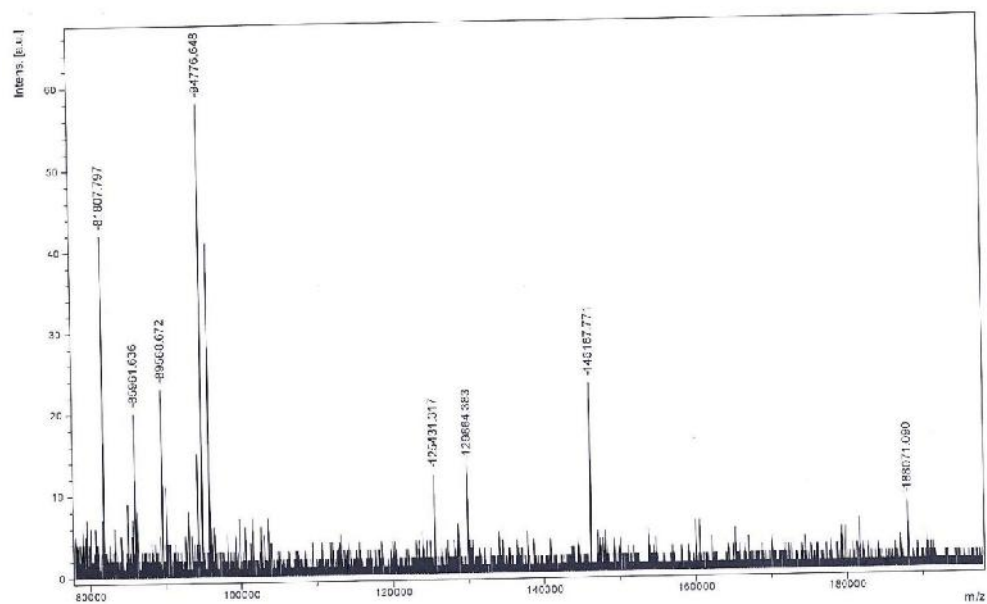


Figure 7S: Tandem mass spectra of GM

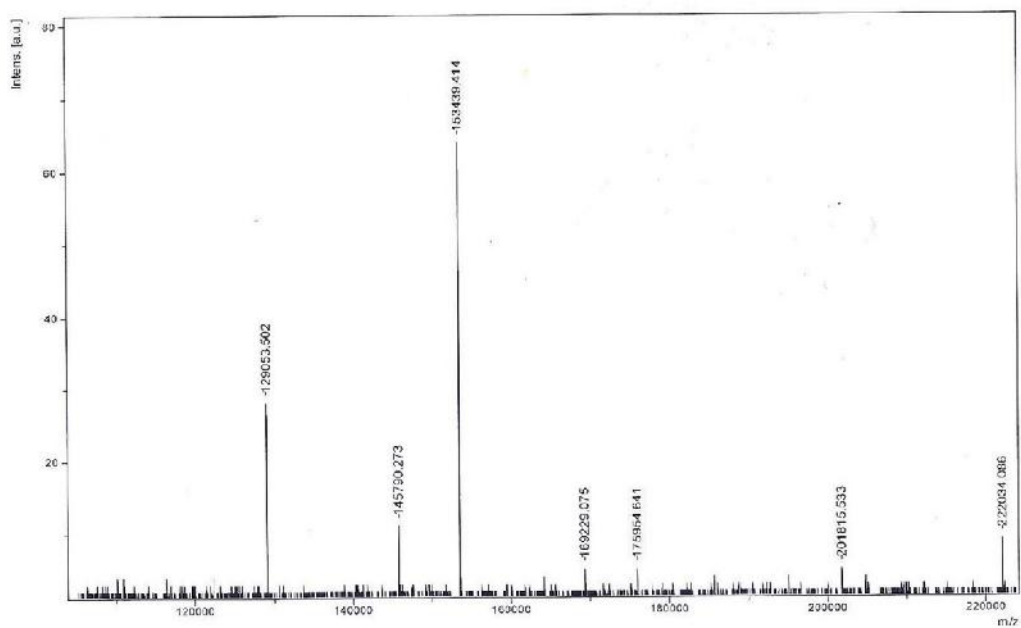


Figure 8S: Tandem mass spectra of GM-OCM

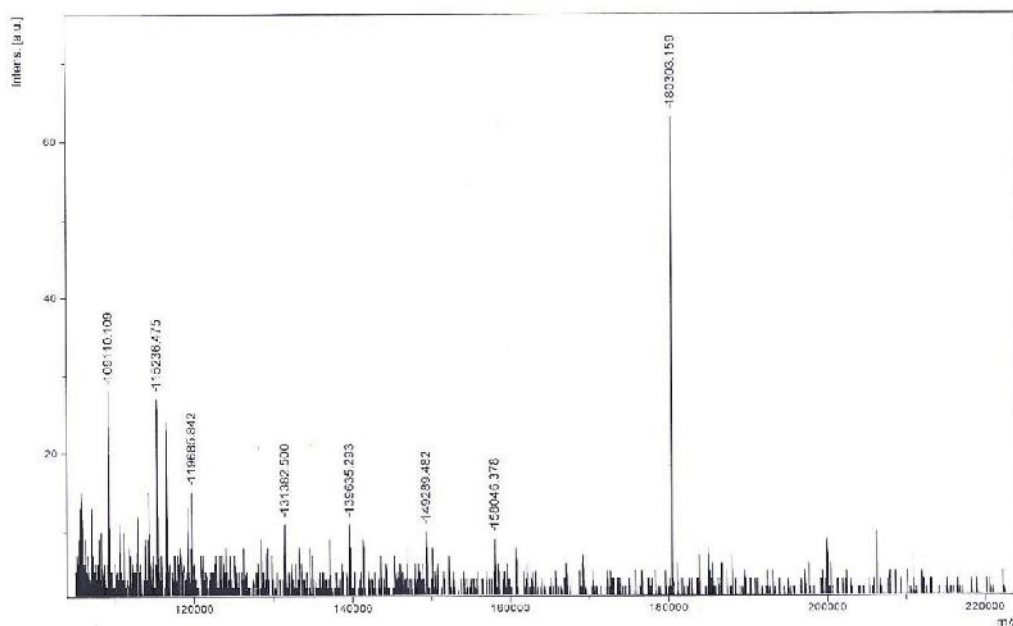


Figure 9S: Tandem mass spectra of GM-OCM-DSPE

2. *In-vitro* ligand binding specificity: ConA agglutination assay

The presence of accessible mannose residues on the surface of GM-bilosomes was confirmed by *in-vitro* Concanavalin A (Con A) agglutination assay. Briefly, 200 μ L of nanoformulations were added to 1 mL of agglutination causing reagent [Con A (1mg/mL) + calcium chloride (5mM) + magnesium chloride (5mM)]. The increase in turbidity was monitored after 2 h of incubation at 550 nm using UV spectrophotometer. A significant increase (Figure 10S) in the turbidity at 550 nm was observed due to presence of mannose or glucose residue on the surface of GM-bilosomes which may be attributed to specific binding of –OH group of monosaccharide with amino acid of Con A. This observation revealed the incorporation of GM-OCM-DSPE in bilosomes and their accessibility to bind to the mannosyl receptors present on the surface of the antigen presenting cells (APCs).

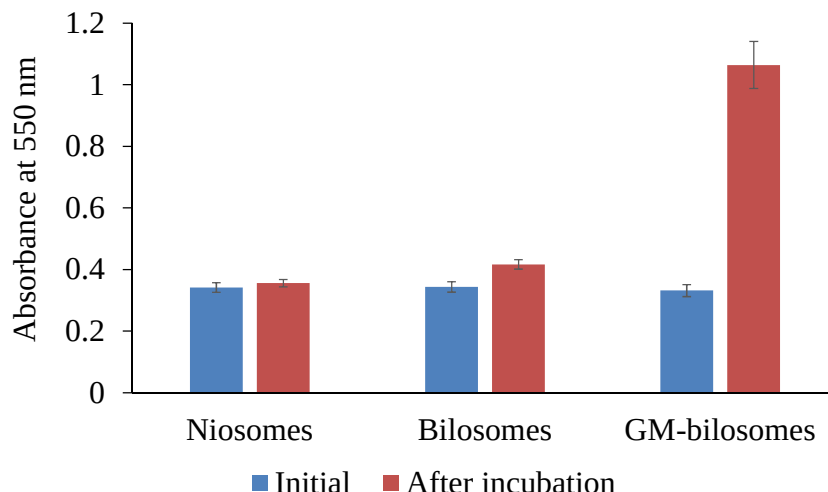


Figure 10S: *In-vitro* ligand specificity of GM-bilosomes by Con A aggregation assay. Data is expressed as mean $\pm$ S.D ($n = 6$).

3. *In-vitro* release study

In-vitro release studies were performed using PBS as release media. Briefly, vesicular formulations (2 mL) were diluted up to 100 mL using PBS (pH 7.4) and incubated at 37°C with gentle shaking at 80 rpm in a shaker bath. Aliquots (0.5 mL) were sampled at 0.5, 1, 2, 3, 4, 6, 8, 12, 18 and 24 h and replaced with equal quantity of fresh medium to mimic the sink condition. The untrapped TT was separated by passing the vesicular dispersion through Sephadex column, vesicles were disrupted using Triton X-100 and the amount extracted was analyzed colorimetric analysis at 561 nm as described above.

The *in-vitro* release profile observed in case of different formulations is shown in Figure 11S. Approximately 20% of the entrapped TT was released in case of GM-bilosomes within first 2 h that approached to approximately 75% in 24 h while it was up to 30% within first 2 h and approached to approximately 87% in case of bilosomes and niosomes. Drug released from GM-bilosomes was significantly ($p < 0.05$) lower in comparison with bilosomes and niosomes while almost similar and insignificant ($p > 0.05$) difference in release profile was observed between niosomes and bilosomes.

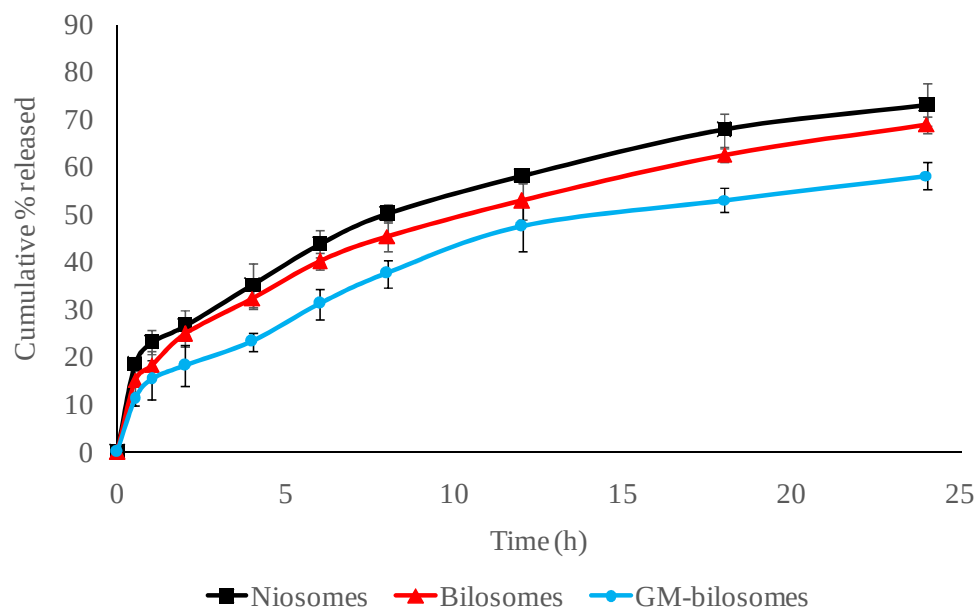


Figure 11S: Comparative *in-vitro* release profile of different vesicular formulations in PBS (pH 7.4)