Mucosal and systemic immune responses following mucosal immunisation of tetanus toxoid entrapped in lipid nanoparticles prepared by microwave reactor

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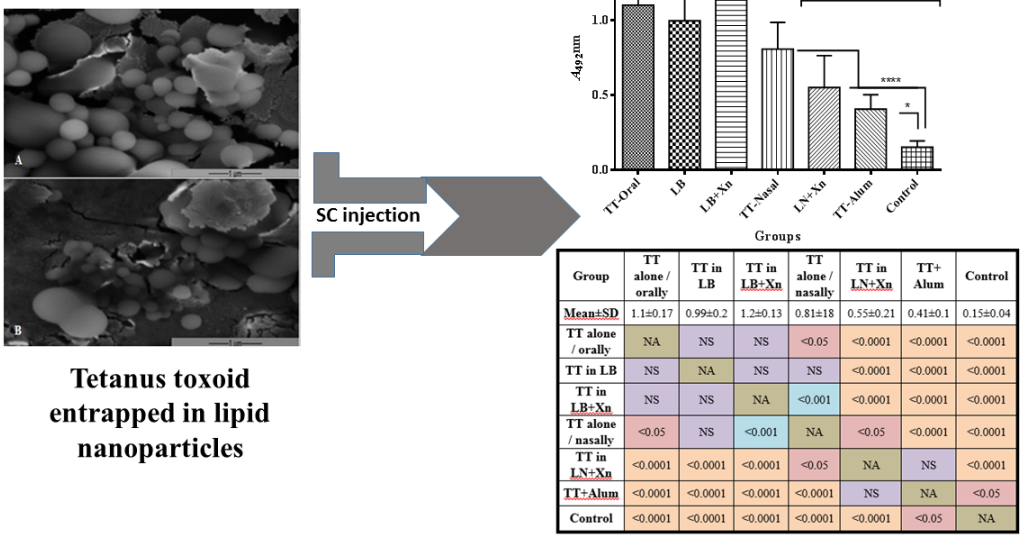
Graphical abstract



Tetanus toxoid entrapped in lipid nanoparticles

Group	TT alone / orally	TT in LB	TT in LB+Xn	TT alone / nasally	TT in LN+Xn	TT+ Alum	Control
Mean±SD	1.1±0.17	0.99±0.2	1.2±0.13	0.81±18	0.55±0.21	0.41±0.1	0.15±0.04
TT alone / orally	NA	NS	NS	<0.05	<0.0001	<0.0001	<0.0001
TT in LB	NS	NA	NS	NS	<0.0001	<0.0001	<0.0001
TT in LB+Xn	NS	NS	NA	<0.001	<0.0001	<0.0001	<0.0001
TT alone / nasally	<0.05	NS	<0.001	NA	<0.05	<0.0001	<0.0001
TT in LN+Xn	<0.0001	<0.0001	<0.0001	<0.05	NA	NS	<0.0001
TT+Alum	<0.0001	<0.0001	<0.0001	<0.0001	NS	NA	<0.05
Control	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.05	NA

Abstract:

In this study, the use of a microwave reactor, which allowed high input of energy into a pressurised system in a short period of time, was investigated for preparation of lipid nanoparticles (LNPs). The aim was to optimise the formulation process by reducing manufacturing time. Two types of LNPs were prepared; non-ionic surfactant vesicles (NISV) and bilosomes (modified NISV incorporating bile salts), with a model antigen (tetanus toxoid, TT) and the immune response induced after mucosal (nasal and oral, respectively) administration was assessed. The TT loaded LNPs were characterised in terms of particle size, size distribution, morphology, and entrapment efficiency. Immunisation was evaluated by lethal challenge with tetanus toxin in an animal model. The efficiency of vaccination was evaluated by measuring the anti-TT IgG antibody levels in the vaccinated animals. Bilosomes formed by this method showed an immunogen entrapment efficiency of ~ 30% which was significantly ($p<0.05$) higher than entrapment

efficiency in the NISV. The percentage of animals that survived when challenged with tetanus toxin correlated with the level of IgG determined in the serum of mice immunised with LNPs by the mucosal route. Moreover, there were significant ($p<0.05$) differences between orally and nasally immunised groups. Animal groups immunised bilosomes via the oral route showed the highest level of IgG (1.2±0.13) compared to the positive control, LN+Xn, and no immunised group. Similarly, groups immunised via the nasal route showed significantly ($p<0.0001$) higher titres compared with the control group. Mucosal TT was capable of inducing systemic specific IgG anti-TT responses that were higher than the parenteral vaccine.

Key words:

Microwave reactor, tetanus toxin, mucosal immunisation, niosomes, and bilosomes.

1. Introduction

Non-ionic surfactant vesicles (NISV) for vaccines delivery are prepared by various conventional bulk methods such as thin-film hydration, reversed phase evaporation, and ethanol injection, which utilise mixing of two liquid phases on a bench scale at elevated temperature, in order to facilitate spontaneous self-assembly of the lipid components into bilayer vesicles. One approach reported by Mozafari *et al*. is the heating method (or referred here as microwave method) [1], in which NISV were prepared without the use of organic solvents, and the various components were hydrated in aqueous media at room temperature followed by heating at 120°C with mechanical stirring [2].

Using microwave reactor, the formation of LNPs is occurred in 1-2mins with ability to scale the production to large volume. Produced LNPs in suspension can be loaded with any antigen or freeze dried and resuspended using the antigen solution. Moreover, is more controlled with higher reproducibility compared to the old traditional film hydration method for LNP preparation. This is common advantage for the microwave reactor with the microfluidic mixing technique for LNP preparation as both can prepare the particles efficiently in a very short period of time. However, microfluidic mixing requires careful control on the mixing parameters such as the total flow rates and the flow rate ratios [3].

For the vast majority of vaccines, the main route of administration used is intramuscular (IM) or subcutaneous (SC) injection, as this has been shown to be effective in eliciting protective immune responses. However, whilst effective, needle-based vaccination comes with a number of disadvantages including risk of needle-stick injuries, associated pain and decreased patient compliance, necessity for delivery by medical professionals and cost of paying them [4, 5]. New routes and methods for vaccine delivery are consequently a major

area of research, and needle-free vaccination is supported by key global players such as the World Health Organisation (WHO), the Global Alliance for Vaccines and Immunization (GAVI) and the Centre for Disease Control and Prevention (CDC) [6, 7].

The other route of administration which research has focussed on has been via the mucosae, with nasal and oral administration regarded as the main sites. Mucosal administration of vaccines is a desirable goal as it enables protection systemically as well as at mucosal surfaces where many pathogens invade their mammalian hosts via respiratory, gastrointestinal and urogenital mucosal surfaces. On the other hand most parenteral vaccines only provide systemic defence, therefore, it is pertinent to elicit mucosal immunity to protect the mucosae [7, 8]. There are a few mucosal vaccines on the market, but these are based on live-attenuated or whole-killed organisms and there are safety concerns with regard to potential virulence reversion [9]. For example, one of the currently licensed mucosal influenza vaccines is FluMist ™ from Medimmune, which is delivered nasally as a spray by a pre-filled single use device [10, 11]. Acellular or subunit immunogens have a better safety profile, however, these components are also generally poorly immunogenic. In addition, choosing the most appropriate mucosal route to induce high efficacy remains a challenge [12].

Oral vaccination remains a more attractive route, as the highest degree of patient compliance and simplicity is expected with patients having used this route for drug administration. Vaccination in this way may be more attractive, however, it requires protection of protein antigens from enzymatic degradation in the gastrointestinal tract (GIT) and a means to overcome poor transition across the gut wall. Therefore, protective carrier systems are required to overcome these hurdles [13, 14].

Furthermore, the efficacy of most vaccines requires enhancement by the use of adjuvants, agents which can increase immune responses; some of which can be used as vaccine delivery systems to allow mucosal immunisation [15, 16]. Particulate based systems, such as liposomes, NISV and bilosomes, are of great interest as they can mimic the particulate nature of viruses. This therefore allows targeting of the antigen-presenting cells of the immune system [17], and the possibility of antigen encapsulation allows transportation and protection of soluble antigens through the GIT.

Previous studies have demonstrated that lipid nanoparticles (LNPs) provide antigen protection as well as inherent adjuvant properties that enhance immune responses [18-20]. In this study, we examined NISV, prepared by microwave reactor, via nasal administration, and bilosomes designed for oral administration [21]. The efficacy of immunisation with a model antigen tetanus toxoid (TT) loaded LNPs was evaluated in a tetanus toxin challenge model [22].

2. Materials and methods

2.1.Materials

Monopalmitoyl glycerol (MPG) was purchased from Larodan AG, Sweden. Cholesterol (Chol), dicetyl phosphate (DCP), sodium deoxycholate (DOC), Xanthum gum (Xn), ninhydrin reagent, sodium dodecyl sulphate (SDS), and bovine serum albumin (BSA) were all obtained from Sigma-Aldrich, UK. TT antigen was produced at Finlay Vaccine Institute (lot L-4003-TET).

2.2. Methods

2.2.1. Vesicle preparation

Empty bilosomes and niosomes (EB and EN, respectively) were prepared using a microwave reactor (MW method). MPG, Chol, and DCP at a molar ratio of 5:4:1 were added to 8ml of carbonate buffer (0.025M, pH 9.7) in a sealed 20ml microwave glass tube with a magnetic stirring bar. For the bilosome preparation, 1ml of carbonate buffer was replaced by 1ml of 100mM sodium deoxycholate solution before adding the lipids. The mixture was irradiated in a Biotage Initiator microwave (Biotage, Upsala, Sweden) for 2min at 140°C under pressure with continuous stirring. After cooling to 30°C, 2ml of antigen solution in carbonate buffer was added slowly with continuous stirring for 1h. The mixture was vigorously vortexed for 5min. The formed emulsion was kept at 4°C until used in immunisation or lyophilised for long-term storage using an Edwards Modulyo freeze drier at -45°C under pressure for 24h.

Lyophilised LNP were reconstituted with different amounts of TT antigen in order to formulate loaded bilosomes (LB) or NISV (LN). The mixtures of LNP:TT were formulated in 1:1 and 10:1 ratios, then vortexed for 5 min to ensure protein entrapment into the vesicles. Xn was added to increase mucoadhesivity, TT-loaded LNPs mixed with a solution of Xn designated LN+Xn and LB+Xn, containing a final Xn concentration of 0.1% (w/v).

2.2.2. Vesicle size and zeta potential (ζ-potential)

Particle sizing and zeta potential (ζ-potential) measurements were determined by dynamic light scattering (DLS) and phase analysis light scattering (PALS), respectively, using a Nano ZS® (Malvern, UK) at 25°C. Each measurement was carried out for three runs and the average taken. Empty LNPs were diluted 1:50 in 0.025M carbonate buffer, pH 9.7 immediately before measurements were taken.

2.2.3. Scanning Electron Microscopy (SEM)

SEM was carried out at the Electron Microscopy Facility, School of Life Sciences, University of Glasgow, UK. Briefly, diluted empty LNP samples were deposited onto a carbon-tape substrate attached to aluminium pin stubs dried, and coated with gold/palladium with a Polaron SC515 sputter coater and imaged on a JEOL 6400 scanning electron microscope with an ADDA3 digital interface at 3-10kV.

2.2.4. Quantification of the protein entrapped within LNP vesicles

Entrapment of protein was quantified using two different methods which are a modified ninhydrin assay, which was reported to be unaffected by lipid interference [23], and a bicinchoninic acid (BCA) based method. For both methods, samples were subjected to ultracentrifugation in order to separate entrapped antigen from free antigen. Briefly, 0.11ml TT-loaded LNPs diluted in 4ml of 0.025M carbonate buffer, pH 9.7, was spun in a Beckman tube in a Beckman XL-90 ultracentrifuge (Beckman RIIC, UK) at 35,000 rpm for 2h. The pellet was resuspended in 0.11ml 0.025M carbonate buffer, pH 9.7, and then transferred to 1.5ml microfuge tubes for the ninhydrin protein quantification assay. The supernatant was collected and antigen concentration determined using a bicinchoninic acid (BCA) assay.

2.2.4.1. Modified ninhydrin assay

The samples, along with standards were prepared with 0.1, 0.25, 0.5, 0.75, 1.0, 1.5 and 2mg of antigen, all in 1.5 ml microfuge tubes, and placed in an oven at 90°C overnight. One hundred and fifty microlitres of 13.5M NaOH was then added to each tube, with a pinhole made in the lid before autoclaving at 121°C/131 kPa for 20min. After removal from the autoclave, the holes were sealed with autoclave tape and the NaOH neutralised with 250µl glacial acetic acid, followed by vortexing for 5s. To each tube, 500µl of

ninhydrin reagent was then added, vortexed and placed in a water bath at 90°C for 20min. Two hundred and fifty microlitres of the resultant mixture was transferred to a fresh tube containing 750µl of 50% (v/v) propan-2-ol and vortexed. Each sample (200µl) was transferred to a flat-bottomed 96-well plate and the absorbance read at 540nm in a microplate reader (SpectraMax M5, Molecular Devices, USA), with test sample levels determined by linear regression from the standard calibration curve.

2.2.4.2. BCA assay

Supernatants obtained from ultracentrifugation were quantified for un-entrapped antigen. Briefly, BSA was dissolved in 0.1M NaOH/1% (w/v) sodium dodecyl sulphate (SDS) to a final concentration of 2mg/ml to prepare a stock standard solution. Twenty five microlitres of each sample was added to 75µl of 0.1M NaOH/1% (w/v) SDS. Samples and BSA were heated in a block set at 90°C for 15min and then allowed to cool before being centrifuged at 13000 rpm for 15min. Ten microlitres of each sample and BSA standard dilutions (125-2000µg/ml) were transferred into a microtitre plate. To each well, 190µl of BCA combined reagent (Thermo Fisher Scientific Inc., UK) was added and the plate was incubated at 37°C for 30min. The absorbance of the samples were read at a wavelength of 560nm in a SpectraMax M5 plate reader. The quantity of antigen in the supernatant was then subtracted from the total antigen amount added to the LNP in order to calculate the entrapment efficiency percentage (EE %).

2.2.5. Evaluation of vaccination efficacy after immunisation with TT loaded LNPs:

The immunisation experiments described below were performed in accordance with UK Home Office regulations and the approval from the local ethical committee. OF1 outbred mice were randomly divided into groups of 10 mice. Control groups were included when

required in each experiment. Mice in the positive control group were immunised with the recommended dose of TT vaccine (vax-TET®). Mice in the negative control group received no immunisation.

2.2.5.1. TT challenge model validation

On day 0, mice were immunised with LB+Xn by the SC route in a single dose as shown in Table 1A to validate the challenge model. On day 49 all groups including the negative control group were injected with LD_{50} tetanus toxin for challenge evaluation. Survivors and deaths were recorded for the next 4 days post-challenge.

2.2.5.2. Mucosal immunisation with TT loaded LNPs

Mice were immunised on day 0 with TT loaded LNPs. LB+Xn and LN+Xn were administered by the mucosal routes referred to in Table 1B. Tail bleeds were collected on day 42 from all groups including the control groups for evaluation of IgG against TT in the serum. On day 28 after the final immunisation at day 21 (28+21 = 49), all groups including the negative control group were injected with LD50 tetanus toxin for challenge evaluation. Survivors and deaths were recorded for the next 4 days post-challenge.

Table 1 Schedule and groups for the *in vivo* challenge experiment with tetanus toxin.

Table 1A (Parenteral route)							
Group (n=10)	**Immunogen**	**Route**	**Dose (LF)**	**Dose (ml)**	**Schedule**	**Sample**	**Challenge**
1	LB+Xn (1:1)	SC	0.1	0.5	Day 0	-	Tetanus toxin LD_{50} injected on day 49
2	LB+Xn (1:1)	SC	0.05	0.5	Day 0	-	
3	LB+Xn (1:1)	SC	0.025	0.5	Day 0	-	
4	LB+Xn (1:1)	SC	0.0125	0.5	Day 0	-	
5	LB+Xn (10:1)	SC	0.1	0.5	Day 0	-	

6	LB+Xn (10:1)	SC	0.05	0.5	Day 0	-	
7	LB+Xn (10:1)	SC	0.025	0.5	Day 0	-	
8	LB+Xn (10:1)	SC	0.0125	0.5	Day 0	-	
9	**vax-TET A**	SC	0.1	0.5	Day 0	-	
10	**vax-TET B**	SC	0.05	0.5	Day 0	-	
11	**vax-TET C**	SC	0.025	0.5	Day 0	-	
12	**vax-TET D**	SC	0.0125	0.5	Day 0	-	
13	Control	SC	No immunisation				

Table 1B (Mucosal route)

Group (n=10)	**Immunogen**	**Route**	**Dose (µg)**	**Dose (ml)**	**Schedule**	**Sample**	**Challenge**
1	TT alone	Oral	200	0.2	Days 0, 7, 14, and 21	Sera at day 42 for IgG ELISA	Tetanus toxin LD_{50} injected on day 49
2	TT in LB						
3	TT in LB+Xn						
5	TT alone	Nasal	50	0.02			
6	TT in LN+Xn						
7	TT+Alum	SC	0.16	0.5	Day 21		
8	Control	No immunisation					

Abbreviations: TT; tetanus toxoid, LB; TT loaded bilosomes, LB+Xn; TT loaded bilosome in 0.1% xanthan gum solution, LN+Xn; TT loaded NISV in 0.1% xanthan gum solution, TT+Alum; tetanus toxoid adsorbed onto an aluminum hydroxide gel (vax-TET®, Finlay institute, Cuba).

2.2.6. Measurement of specific antibody levels

Evaluation of anti-TT IgG antibody was carried out by direct ELISA using polystyrene 96-well plates. Briefly, plates were coated with TT (100µl per well) at 5µg/ml in carbonate buffer (0.1mol/l, pH 9.6) at 4°C overnight, and blocked with 1% (w/v) BSA in PBS (0.15mol/l, pH 7.3, blocking solution) for 1h at room temperature. Serum samples were diluted 1:100 in blocking solution and incubated for 1h at 37°C. Anti-mouse IgG peroxidase-conjugated antibodies (Sigma) were added (100µl per well) at 1:2500 dilution in blocking solution and incubated for 1h at 37°C. Bound antibodies were detected with 100µl per well of a substrate–chromogen mixture (o-phenylenediamine and H_2O_2 in citrate–phosphate buffer, pH 5). The reaction was stopped by adding 50µl of H_2SO_4 at

2mol/l and the optical density at 492 nm was measured in a microplate reader (Titertek, Multiskan Plus; Labsystem). All incubation steps were followed by three washes with PBS containing 0.05% (v/v) Tween-20.

2.2.7. Statistical analysis

Statistical significance and differences were compared against the control group, determined by one-way ANOVA with Dunnett's post-test performed using GraphPad Prism version 5.0 for Windows (GraphPad Software, USA).

3. Results

3.1 Vesicle sizing/ zeta potential

Measurement of particle size and ζ-potential values of formulated LNP are shown in Table 2. Loaded LNP showed a significantly larger mean size than empty ones ($p<0.05$ and $p<0.01$ for bilosoms and NISV, respectively). The ζ-potential values recorded for all formulations indicate that they were negatively charged and in the range of -90 to -120mV.

Table 2 Size, ζ-potential values, and EE% for the test lipid nanoparticles (LNP).

Particles	Size (nm)±SD	PDI	ζ-potential (mV)	EE%
EN	320±80	0.46	-116±17	NA
LN	506±186	0.71	-104±9	24±2.2
EB	236±68	0.64	-109±12	NA
LB	316±14	0.44	-96±7	31±6.8

Abbreviations; PDI: Polydispersity index, EB: empty bilosome, LB: loaded bilosome, EN: empty NISV and LN: loaded NISV.

3.2 LNP microphotography by SEM

There was no difference observed between images of EN or EB. Both formulations contained nanoparticles that are spherical structures without lipid sheets or crystalline bodies, with a diameter range from 150nm to 2microns (Figure 1).

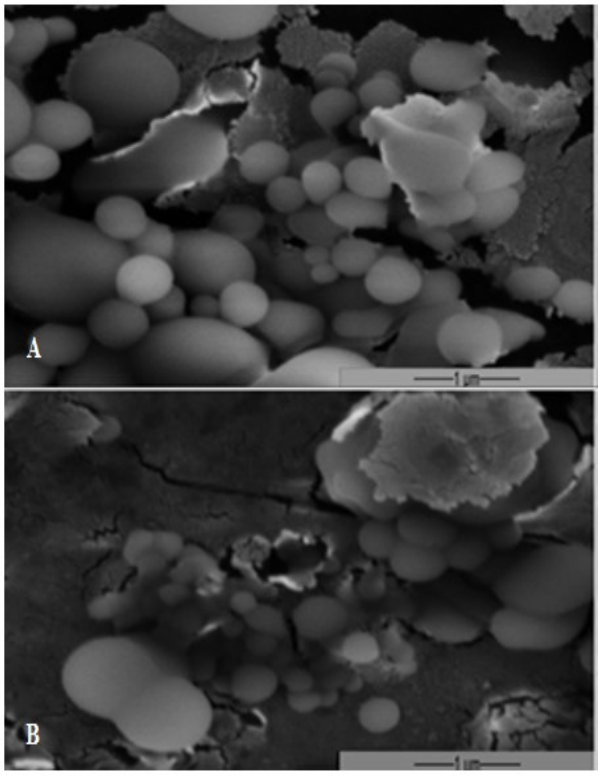


Figure 1 Scanning electron micrograph showing typical range of (A) EB and (B) EN. Samples were coated with gold/palladium with a Polaron SC515 sputter coater and imaged on a JEOL 6400 scanning electron microscope with an ADDA3 digital interface at 3-10kV to enhance surface imaging.

3.3 Estimation of antigen entrapment percentage

The ninhydrin assay showed that the range of antigen loaded into LNP was between 22-38% of the original amount of protein added to the mixture. EE% was significantly higher in the bilosome than in the NISV ($p<0.05$, Table 2). Determination of protein concentration

by BCA assay in the supernatant after ultra-centrifugation confirmed the EE% observed by ninhydrin assay.

3.4 Survival rate in TT challenge experiment and antibody response

A pilot challenge study was carried out with LD_{50} tetanus toxin injected into mice to validate the assay and determine the dose response and LNP:immunogen ratio compared to standard vaccine vax-TET (Table 3). Mice were monitored regularly; deaths were recorded and at the first sign of paralysis were sacrificed and designated not protected. Mice that showed no sign of paralysis for over 4 days were designated protected against lethal toxin challenge. The optimal LNP:immunogen ratio was with the 10:1 formulation and TT dose equivalent to the standard vaccine dose, therefore the next challenge experiments and immunisation were carried out at this ratio.

Table 4 shows the death and final percentage of survivors in the second challenge experiment with the groups immunised by the mucosal route. All animals in the orally immunised groups survived the challenge with no deaths after 4 days. Similarly, 100% of animals in the positive group survived, compared with 80% of animals in the nasally immunised groups and 0% in the negative control group.

The O.D. at 492mn of specific anti-TT IgG antibody was determined in sera of groups vaccinated by the mucosal route (Figure 2). Groups immunised via the oral route showed the highest level of IgG which was significant ($p<0.0001$) compared to the positive control, LN+Xn and no immunised group. Similarly, groups immunised via the nasal route showed significantly higher ($p<0001$) titres compared with the control group. Table 5 shows the IgG OD levels and significant degree between the experimental groups.

Table 3 Survival percentage of mice using various LNP:immunogen ratios and doses via the SC route. N represent the number of animals per group.

Groups				% Survivors				
Oral	LNP:Imm	Dose (LF)	N	Day 0	Day 1	Day 2	Day 3	Day 4
LB+Xn	1:1	0.1000	10	100	100	100	100	90
LB+Xn	1:1	0.0500	10	100	100	100	100	81
LB+Xn	1:1	0.0250	10	100	70	70	40	10
LB+Xn	1:1	0.0125	10	100	0	0	0	0
LB+Xn	10:1	0.1000	10	100	100	100	100	100
LB+Xn	10:1	0.0500	10	100	100	100	100	100
LB+Xn	10:1	0.0250	10	100	100	100	100	100
LB+Xn	10:1	0.0125	10	100	100	100	100	100
vax-TET	**Dose (LF)**							
A	0.1000		10	100	95	95	95	95
B	0.0500		10	100	95	95	85	80
C	0.0250		10	100	95	65	30	20
D	0.0125		10	100	50	20	5	0
Placebo	-		10	100	0	0	0	0

Abbreviations: LB+Xn; loaded bilosome in 0.1% (w/v) xanthan gum solution, LF; flocculation value (0.05 LF= 0.16µg). LNP; lipid nanoparticles, Imm; immunogen protein.

Table 4 Deaths and survival percentage recorded in animals immunised via the mucosal route after challenge with tetanus toxin (International standard).

Group	Route	Dose µg	Animal (N)	Day 1	Day 2	Day 3	Day 4	% Survivors
TT alone	Oral	200	9	0	0	0	0	100
TT in LB			7	0	0	0	0	100
TT in LB+Xn (10:1)			7	0	0	0	0	100
TT alone	Nasal	50	10	1	1	0	0	80
TT in LN+Xn (10:1)			10	1	1	0	0	80
TT+Alum	SC	0.16	10	0	0	0	0	100
Control	No immunisation		10	5	5	-	-	0

Abbreviations: TT; tetanus toxoid, LB; TT loaded bilosomes, LB+Xn; TT loaded bilosome in 0.1% (w/v) xanthan gum solution, LN+Xn; TT loaded NISV in 0.1% (w/v) xanthan gum solution, TT+Alum; tetanus toxoid adsorbed onto an aluminum hydroxide gel (vax-TET®, Finlay Institute, Cuba).

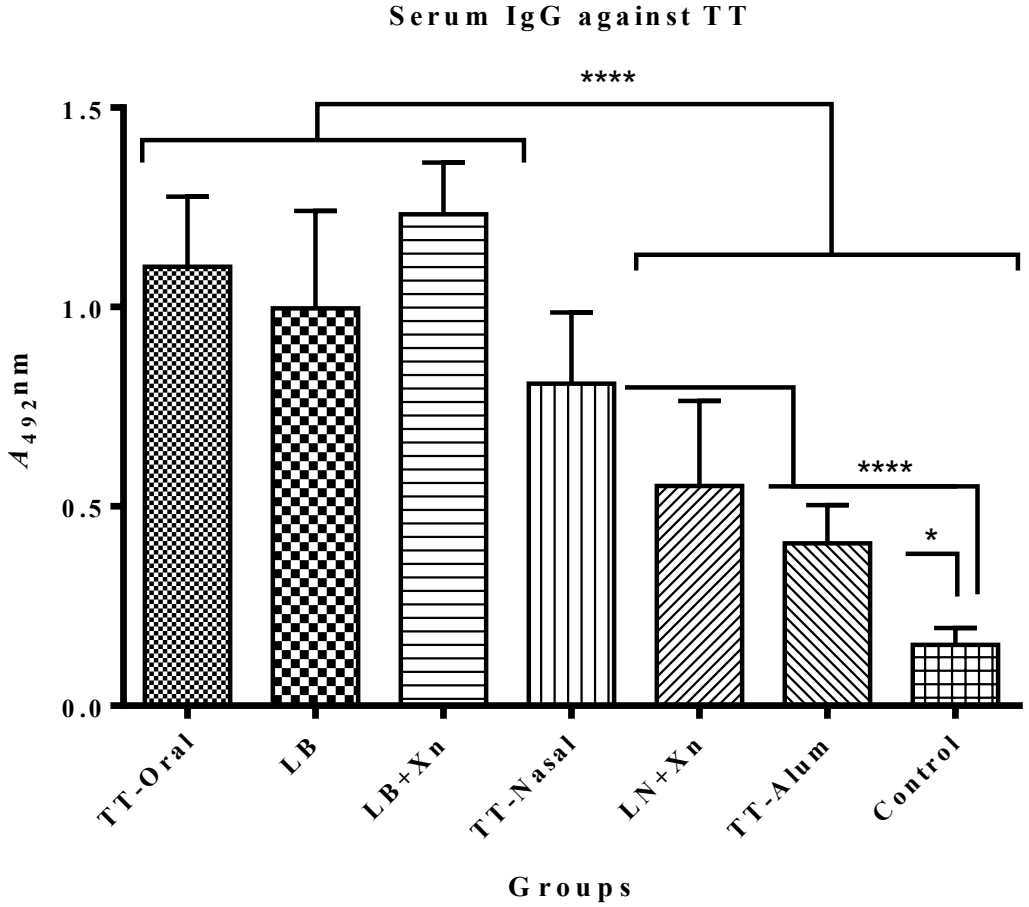


Figure 2 Anti-TT IgG antibodies in sera of mice (n=10) immunised against TT on days 0, 7, 14 and 21. Error bars represent the Mean±SEM.

Abbreviations: TT; tetanus toxoid, LB; TT loaded bilosomes (10:1), LB+Xn; TT loaded bilosome in 0.1% (w/v) xanthan gum solution (10:1), LN+Xn; TT loaded NISV in 0.1% (w/v) xanthan gum solution (10:1), TT+Alum; tetanus toxoid adsorbed onto an aluminum hydroxide gel (vax-TET®, Finlay Institute, Cuba).

Table 5 Mean±SD of anti-TT IgG ELISA reading at 492nm (n=10), and the significant level between groups using ANOVA with Tukey's multiple comparisons test.

Group	TT alone / orally	TT in LB	TT in LB+Xn	TT alone / nasally	TT in LN+Xn	TT+ Alum	Control
Mean±SD	1.1±0.17	0.99±0.2	1.2±0.13	0.81±18	0.55±0.21	0.41±0.1	0.15±0.04
TT alone / orally	NA	NS	NS	<0.05	<0.0001	<0.0001	<0.0001
TT in LB	NS	NA	NS	NS	<0.0001	<0.0001	<0.0001
TT in LB+Xn	NS	NS	NA	<0.001	<0.0001	<0.0001	<0.0001
TT alone / nasally	<0.05	NS	<0.001	NA	<0.05	<0.0001	<0.0001
TT in LN+Xn	<0.0001	<0.0001	<0.0001	<0.05	NA	NS	<0.0001
TT+Alum	<0.0001	<0.0001	<0.0001	<0.0001	NS	NA	<0.05
Control	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.05	NA

Abbreviations: TT; tetanus toxoid, LB; TT loaded bilosomes, LB+Xn; TT loaded bilosome in 0.1% (w/v) xanthan gum solution, LN+Xn; TT loaded NISV in 0.1% (w/v) xanthan gum solution, TT+Alum; tetanus toxoid adsorbed onto an aluminum hydroxide gel (vax-TET®, Finlay Institute, Cuba), NS; not significant.

4. Discussion

In this study, the use of a microwave reactor, which allowed a high input of energy into a pressurised system in a short period of time, was investigated for NISV and bilosome formations. These LNP had a greater range of sizes, most likely due to a lack of homogenisation step to reduce the PDI. The range of sizes was within that expected from previous reports in the literature [24-29]. Confirmation of these sizes was achieved using SEM, which showed spherical particles and the mean sizes were not significantly different from those determined by DLS or previously reported [30, 31].

ζ-potential in all cases were approximately -100mV, as has been previously found with the other preparation methods such as the heating method that used the same lipid components [30]. The negative charge in these formulations was attributed to the use of DCP. Moreover, bilosomes formed using this MW method showed an entrapment efficiency of ~ 30%.

Microwave reactors have previously been used to formulate surfactants which may be used in vesicles [32], and in the production of vaccines [33]. As far as we are aware, it has not been used in the formulation of lipid vesicles. The process described here is therefore a novel method of vesicle formation, and as such, few direct comparisons exist. The effect of microwave energy on lipid vesicles has previously been examined, and it is accepted that this can lead to an increase in membrane permeability [34, 35]. As there is no exposure to microwave radiation after formulation, and given the low exposure time (1min), it is likely that this effect would be temporary. This could easily be determined in future work, by examining the relative release rates of a fluorescent molecule such as carboxyfluorescein from formulations prepared with the MW method and other described

methods in the literature such as the heating method which has previously been used in such studies [36-38]. The increased permeability of the vesicle on exposure to microwave radiation may actually be beneficial in the formulation process, as it could reasonably be expected that this would work in both directions. This could mean that exposure of the formulation after the addition of antigen, at a certain power level, which would not damage the antigen, would allow an equilibration of antigen within and outwith the vesicles. If the volume of the vesicles within a formulation was 50%, then this could possibly allow an entrapment efficiency of 50%, assuming the vesicles hardened after the removal of the radiation.

The dose-response regime for parenteral immunization, controls and challenge experimental designed were selected according the recommendations for evaluation and quality control of Tetanus vaccines [39].

The dosage regime for mucosal immunization was selected based on previous result from the group [40]. Also, the results from other researchers were reviewed. Hagiwara et al. used three doses of 25 ug of tetanus detoxified protein by nasal route combined with cholera toxin adjuvant and obtained 100% protection in the challenge model [41]. Overall, the results from Baljer et al demonstrated that high doses of tetanus toxoid administered by mucosal route in mice may protect in challenge experiment. The author immunized the mice with three doses of Tetanus by oral route 200 Lf (equivalent to 600 ug of protein) each time [42]. In the experiment described in our work we use 3 times less than the dose proposed by the Baljer et al but its still protective in the mice TT challenge model. There is still needed to demonstrate the median lethal dose (LD50) using this TT challenge model and mucosal route.

For lethal challenge experiments, the model was firstly evaluated with vax-TET positive control vaccine. Different ratios of LB+XN particles loaded with TT were formulated and evaluated by the SC route.

The results showed that a 10:1 LNP:immunogen ratio gave the best protection (100%) against toxin challenge compared to the equivalent dose used in the positive control vaccine group (Table 3). Therefore, this result suggests that the use of LB+Xn in a 10:1 ratio formulation has a strong adjuvant effect.

Administration of LNP mucosally demonstrated that the percentage of animals that survived when challenged with tetanus toxin correlated with the level of IgG determined in the serum. Even though, there was no significant difference between orally immunised groups, there were significant differences between orally and nasally immunised groups, perhaps because a high dose was administered orally in comparison to nasally immunised groups. On the other hand, TT alone, administered nasally or orally was significantly higher from the parenteral Alum vaccine (positive control). Mucosal TT was capable of inducing systemic specific IgG anti-TT responses that were higher than the parenteral vaccine. In a previous study, the co-administration of cholera toxin as an adjuvant with TT via the oral route, induced peak serum IgG and IgA anti-TT responses [43, 44]. However, no differences were observed in the experiments between groups immunised with TT alone or with LNP. This raises the question, do LB, LB+Xn and LN+Xn have any adjuvant effects to enhance response to the TT antigen? In any case, the amount of TT administered by the mucosal route is much higher than that established for the parenteral lethal challenge model and further studies will be needed to find the effective dose of TT that satisfies the

prerequisites of the model to protect 50% of animals. Any mucosal adjuvant developed would need to improve this response.

Previous work from Baljer *et al.* applied 3 doses of 200 Lf toxoid (200 Lf are equivalent to approximately 600 µg) and here we used much lower amounts for oral immunisation in our studies, 200 µg which is equivalent to 60 LF. Moreover, we also found that 50 µg of TT (15LF) by intranasal immunization protected 80 % of animals, whereas Baljer *et al.* reported 100% protection with one single intranasal administration of 100LF of tetanus which approximately corresponds to 300 µg [42]. Therefore, the work presented here uses lower doses than reported in the literature and demonstrate that even lower doses for oral or nasal may be used.

The results showed that administration of this antigen alone by the oral route produced systemic and local immune response. The antibodies were specific and protected the animals against toxin challenge. Although no adjuvant effect was demonstrated, it is probable that bilosome-loaded antigens liberated from bilosome vesicles in the GIT have been sampled by antigen-presenting cells (APCs) from the gut-associated lymphoid tissue (GALT) to produce the evaluated immune protective effect.

Immune responses following oral administration have previously been shown to be possible with liposomes and bilosomes, for a range of different antigens such as cholera toxin B, TT, peptide and protein [27, 45-48]. Work by Alves *et al.* (2008) indicated that different degrees of immune response, from immunisation to oral tolerance, are induced depending on both the nature of the liposomes, and the breed of mouse [29, 49].

4. Conclusion

In this study, a microwave reactor was used to prepare lipid vesicles in an innovative method for forming vesicles. After immunisation, there were significant differences between orally and nasally immunised groups, but primarily it was demonstrated that mucosally administered TT is protective against lethal challenge. Furthermore, the results showed that 10:1 LNP:immunogen ratio gave the best protection (100%) against challenge compared to the equivalent immunogen amount used in the positive control.

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