Examination of the effect of niosome preparation methods in encapsulating model antigens on the vesicle characteristics and their ability to induce immune responses

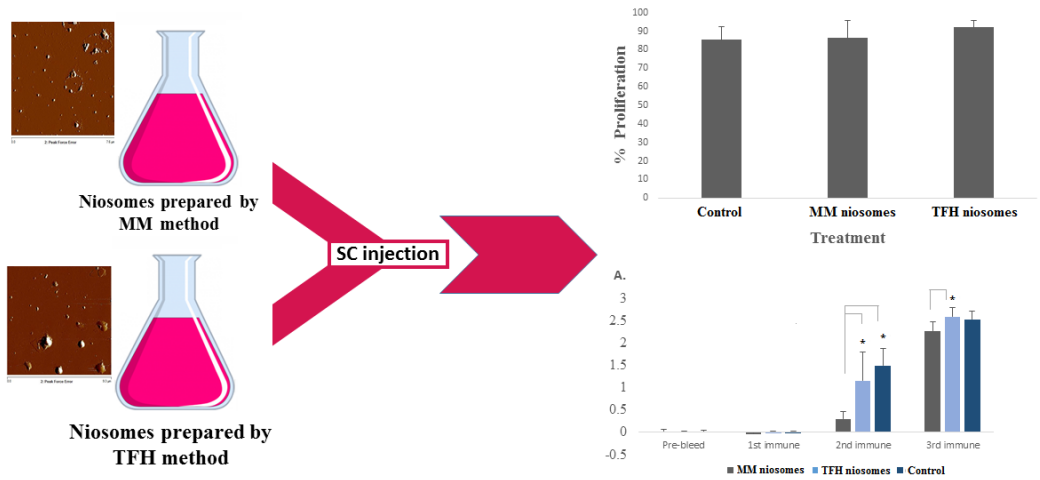
Mohammad A. Obeid[1]*, Teeranun Teeravatcharoenchai[2], David Connell[2], Kanidta Niwasabutra[2], Muattaz Hussain[2], Katharine Carter[2], Valerie A. Ferro[2]

[1] Department of Pharmaceutical Sciences, Faculty of Pharmacy, Yarmouk University, Irbid, Jordan.

[2] Strathclyde Institute of Pharmacy and Biomedical Sciences, University of Strathclyde, 161 Cathedral Street, G4 0RE Glasgow, United Kingdom.

* Corresponding author. E-mail Address: m.obeid@yu.edu.jo ,Tel: 00962798270457

Graphical abstract



Abstract

Niosome nanoparticles can be prepared using different methods, each of which can affect the size and homogeneity of the prepared particles. The aim of this study was to establish if the method of preparation impacted on the prepared vesicles when loaded with a model protein and the type of immune responses induced to the vaccine antigen. Niosomes were prepared using both the traditional thin film hydration (TFH) technique and the microfluidic mixing (MM) technique. Influenza antigen was then entrapped in the niosomes and formulations tested for their ability to induce in *vivo* immune responses in immunized BALB/c mice. Niosomes prepared by MM had a mean size of 157 ± 1.8 nm and were significantly more uniform compared with the niosomes prepared using TFH (mean size 388 ± 10 nm). Niosomes play a key role as an adjuvant to help raise high antibody immune responses. This was confirmed in this study since animals treated with both types of niosomes and antigen were more responsive than unentrapped (free) antigen. Cytokine analysis showed that the TFH niosomes induced a Th1 immune response by raising IgG2a and high levels of IFN-ɣ, while the MM niosomes induced a Th2 immune response by inducing IgG1 ($p<0.05$).

These results confirmed that the method of preparation of the niosomes nanoparticles induced different immune responses and the average particle size of the niosomes differed depending on the method of manufacture. This indicates that particle size and uniformity are of importance and should be taken into consideration when designing an oral based delivery system for vaccine delivery.

Abbreviations

TFH: Thin film hydration, MM: Microfluidic mixing, APC: Antigen presenting cells, MHC: Major histocompatibility complex, BCA: Bicinchoninic acid, ELISA: Enzyme-linked immunosorbent assay, IFN-ɣ: Interferon gamma, Ig: Immunoglobulin, IL: Interleukin, LAIV: Live-attenuated influenza vaccines

1 Introduction

Lipid based nanoparticles include many types such as liposomes, niosomes and micelles. Liposomes are composed of phospholipids as a bilayer structure which can be used for the delivery of different active drugs and antigens. This technology can enhance immunity as an adjuvant by increasing absorption of antigen in addition to holding multiple epitopes of antigen (1). As a result, antigen is protected within the biological environment and has an effect on antigen uptake into the cell leading to endocytosis of antigen by macrophages (1). Niosomes are one type of lipid-based nanoparticles composed of non-ionic surfactants, instead of phospholipids in liposomes, which provide chemical and biological stability for the nanoparticle system (2).

Similar to liposomes, niosomes consist of a bilayer structure surrounding an aqueous compartment (3). Therefore, they can entrap both hydrophilic and lipophilic drugs. In most cases, niosomes contain 3 main components; non-ionic surfactants, cholesterol, and charged molecules (4). The presence of hydrophilic head groups and hydrophobic tails in

the non-ionic surfactant structure help in the formation of bilayers (3, 5). Cholesterol is incorporated into the bilayer structure to stabilise the system and abolish the gel to liquid phase transition of niosomes which leads to less leakiness thus decreasing the release rate of encapsulated material and increasing rigidity of the membrane (6).

Niosomes have been used as a delivery system for various vaccines including influenza antigen (3, 7). Moreover, niosomes with inactivated vaccines have several beneficial aspects in pharmaceutical products. First, they are safe because they cannot replicate to produce disease as in the case of viral delivery systems. In addition, they can elicit an improved immune response greater than the free un-encapsulated inactivated vaccines (8). Moreover, they can provide protection for the encapsulated vaccine, prolong circulation time, reduce toxicity, control drug release, and enhance cell specificity of delivery (9). For lipid based nanoparticles, there are three main considerations when designing them for vaccine delivery: particle size, method of manufacturing and route of administration (10). Particles can vary in size with a diameter between 1 and 1000 μm, referred to as microparticles, whereas nanoparticles have a range from 1 to 1000 nm (11). Generally, small particles are thought to be more effective than large particles, because small ones can penetrate biological barriers and are stabilised in blood circulation. However, there are optimum size ranges for induction of immune responses in a vaccine delivery system. Several studies reported the effect of nanoparticle size on the resulting immune response (10, 12). For example, it has been found that nanoparticles with an average size of 40 nm are suitable for ovalbumin immunisation of mice which can induce both antibody and cellular immune responses (13, 14). Moreover, in an influenza bilosome study, different T cell responses could be induced using different sizes of vesicles. Large

vesicles (400-2500 nm) were found to induce Th1 responses, whereas small vesicles (10-100 nm) induced Th2 responses following oral administration of antigen (15). The reason for these findings was that the large vesicles were thought to induce the release of antigen leading to Th1 responses, while, on the other hand, small vesicles can penetrate M cells and in consequence elicit Th2 responses by dendritic cells found in Peyer's patches (16).

In reference to specific particle size, another study for parenteral administration of niosomal formulation of antigen confirmed that large vesicles with a diameter more than 225 nm can induce Th1 responses, whereas small vesicles less than 155 nm elicit a Th2 response (17). Similarly, data from Conacher *et al*. in parenteral administration showed large vesicles of 3 µm diameter can produce Th1 responses (18). Moreover, our previous work on small vesicles ranging from 50-250 nm where shown to induce Th2 responses after oral administration (19).

Niosomes can be prepared using different manufacturing methods such as the thin film hydration method (TFH), heating method, ether injection method, microfluidic mixing (MM), and many others (2). Different methods will end up in the preparation of vesicles with different characteristics in terms of particles size, lamellarity, and loading efficiency (14). The preparation of niosomes using the thin film hydration method involves dissolving the lipid components in an organic solvent such as chloroform and then evaporating the solvent under reduced pressure using a Rota-evaporator to form a thin layer of the lipid components on the wall of a round bottom flask. Niosomes are then formed after the addition of the aqueous phase at a temperature above the phase transition of the lipids used (2). Niosomes prepared using this method are generally large and

multilamilar which usually require a further size reduction step (2). Microfluidic mixing on the other hand enables the production of small and uniform sized vesicles in one step without the need for post-manufacturing size reduction (20). This method involves mixing of the lipids components dissolved in ethanol, with the aqueous phase at a controlled rate and ratio between the two phases to produce small particles in a desired nanoscale size (6).

Therefore, the aim of this study was to confirm whether the niosomes preparation method can have effect on the types of T-cell immune response. Two different methods were used in the preparation of niosomes, TFH, with post-processing using a mini-extruder, which was used to prepare large niosomes and MM which was used to prepare small niosomes. Albumin or influenza antigen were encapsulated into the prepared niosomes. Vesicles prepared using albumin or influenza antigen were characterised on the basis of their size, polydispersity, and surface charge. Mice then were immunised with influenza antigen loaded niosomes and antibody and cytokine analysis were carried out.

2 Materials and methods

1.1 Materials

1 µm Nucleopore Track-Etch Membrane (GE Healthcare, UK), 4-Nitrophenyl phosphate disodium salt hexahydrate (Sigma Aldrich, Germany), AKP Streptavidin (BD Biosciences, UK), Albumin, from chicken egg white (Sigma Aldrich, USA), Biotin mouse anti-rat IL-4 (BD Biosciences, UK), Biotin rat anti-mouse IFN-ɣ (BD Biosciences, UK), Blocking agent in PBS (Superblock®) (Thermoscientific, USA), Cholesterol (Chol) (Sigma Aldrich, USA), Dicetyl phosphate (DCP) (Sigma Aldrich, Germany), Dried

skimmed milk powder (Marvel, UK), Fold capillary cells (Malvern, UK), Goat anti-mouse IgG1: HRP (Bio-rad, UK), Goat anti-mouse IgG2a: HRP (Bio-rad, UK), Influenza (H3N2*) A/Switzerland/9715293/2013* haemagglutinin (HA) (GPO, Thailand), MicroCentaur (MSE, UK), Microplate BCA Protein Assay Kit (Pierce Biotechnology, USA), Monopalmitin (Monohexadecanoin) (MPG) (Larodan Fine Chemicals AB, Sweden), Phosphate buffered saline (Dulbecco A) (Oxoid, UK), Purified mouse anti-rat IL-4 (BD Biosciences, UK), Purified rat anti-mouse/anti-human IFN-ɣ (BD Biosciences, UK), Resazurin sodium salt (Sigma Aldrich, USE), Sulphuric acid solution (Sigma Aldrich, Germany), TMB Stabilized chromogen (Invitrogen, USA), TWEEN® 20 polyoxyethylene sorbitan monolaurate (Sigma Aldrich, USA).

2.2 Preparation of niosomes by the thin-film hydration (TFH) method

Niosomes composed of MPG, Chol and DCP at 5:4:1 molar ratio were prepared by the TFH method as described previously (2). Briefly, the lipid components were dissolved in chloroform to make a 15% (w/v) lipid in organic solvent mixture in a round-bottom flask. The flask was shaken until a clear solution was obtained, then it was connected to a rotary evaporator (Rotavapor R-3, BTECH, Switzerland) operated at 50 rpm under vacuum at 50 ºC until complete evaporation of chloroform occurred and the formation of a thin film of lipid was observed on the flask wall. Influenza antigen or albumin at a concentration of 60 µg/ml in phosphate buffered saline (PBS, pH 7.3) was warmed at 50ºC and then added to the lipid film and mixed using the rotary evaporator (without vacuum) in order to produce multilamilar vesicles. Next, these vesicles were passed through a mini-extruder with a 1µm pore diameter polycarbonate (PC) membrane (Avanti polar lipids, Alabaster,

AL, USA) at 50 ºC on a hot plate for 21 passages to reduce the particle size and polydispersity. These niosomes were then centrifuged at 18000g for 4.5h to remove un-entrapped antigen. The supernatant was collected and analysed for protein content and the pellet was re-suspended in the same volume as the supernatant.

2.3 Preparation of niosomes using microfluidic mixing (MM)

Niosomes were prepared by MM using a NanoAssemblr^{TM} (Benchtop, Precision NanoSystems Inc., Vancouver, Canada) as described previously (20). This machine has two inlets and one outlet. Niosomes were prepared by mixing the lipid phase (MPG, Chol, and DCP at 5:4:1 dissolved in ethanol 15 % (w/v)) with the aqueous phase (PBS containing influenza antigen or albumin at a concentration of 72 µg/ml) at 3:1 aqueous: ethanol ratio with a total flow rate of 12 ml/min at 50ºC. Niosomes were collected from the outlet and the solution was centrifuged as in section 2.2.

2.4 Measurement of vesicle size, polydispersity, and zeta potential

Particle size, poly dispersity index (PDI), and Zeta potential (ZP) of the MM and the TFH niosomes were measured using a Zetasizer Nano-ZS (Malvern Instruments, UK). The measurements were carried out for both niosomes formulations at 25 ºC diluted 1 in 10 in PBS. All samples were prepared in triplicate and the average particle size ($Z_{Average}$), PDI and ZP reported.

2.5 Imaging the niosomes by Atomic Force Microscopy (AFM)

Morphological examination of both niosome formulations was performed by atomic force microscopy (AFM). Each formulation (5 -10µL) was deposited onto freshly cleaved mica surfaces (G250-2 Mica sheets 1” x 1” x 0.006”; Agar Scientific Ltd., Essex, UK), and left

in to air dry for 1 h before AFM imaging. The images were obtained by scanning the mica surface in air under ambient conditions using a Dimension FastScan BioAFM (Bruker, CA, USA) operated on Peak Force QNM mode. The AFM measurements were obtained using ScanAsyst-air probes. AFM images were collected by random spot surface sampling (at least three areas). The analyses were performed using the Nanoscope Analysis v1.4 (Bruker, USA).

2.6 Niosome entrapment efficiency

A bicinchoninic acid (BCA) assay kit was used to determine protein concentration using the manufacturer's instructions. The Pierce BCA assay is based on the reduction of cupric ions to cuprous ions by protein in an alkaline medium and BCA detects the cuprous ions. However, some substances can also reduce copper such as disulphide reducing agents. Therefore, compatibility reagent as provided in the kit is required to minimise interference. The range of albumin standard was 15-1000 µg whereas the concentration of influenza antigen was 5-285 µg. Standards were aliquoted into duplicate wells, while samples and PBS (blank) were analysed in triplicate in a 96 well microplate (9 µL/well). Four µL of compatible reagent solution was added to each well. The plate was occluded from light using aluminium foil and placed on a plate shaker at a medium speed for one min, followed by incubation at 37ºC for 15 min. Then, 260µL of working reagent was added to each well, the plate was shaken on a plate shaker for one minute, incubated at 37ºC for 30 min and then left at room temperature for 5 min. The absorbance was read at 562 nm. The mean absorbance of the blank wells was subtracted from the mean values of the samples and standards.

The percentage entrapment efficiency was calculated using the following equation:

$$\% \text{ entrapment efficiency} = \frac{\text{Total concentration} - \text{Supernatant concentration}}{\text{Total concentration}} \text{ x } 100$$

2.7 Immunisation study

The *in vivo* experiments described below were performed in accordance with UK Home Office regulations and had ethical approval from the University of Strathclyde. Experiments were carried out in accordance to ARRIVE guideline. In-house bred female BALB/c, 18 weeks old mice were housed in groups of three at 19°C to 23°C with a 12-h light-dark cycle. They were fed a conventional diet (Rat and Mouse Standard Expanded, B&K Universal, UK), with mains water provided *ad libitum*. Mice were immunised by subcutaneous administration in weeks 0, 2 and 4 with 0.1 ml formulation (Table 1 for treatment groups). All mice were injected in the morning with 15 µg influenza antigen per dose. Serum was prepared from tail bleeds, pre-immunisation and three days post-immunisation, by centrifuging at 13000 rpm for 5 min. The serum was stored at -20 ºC until analysed by enzyme-linked immunoassay (ELISA).

Table 1 Treatment groups with different formulations

Group	Mouse no	Formulation
A	1-5	MM niosomes + antigen
B	6-10	TFH niosomes + antigen
C	11-15	Control (antigen only)

2.8 Enzyme-linked immunoassay (ELISA)

Half of a 96 well plate was pre-coated the day before with influenza antigen 0.4 μg in PBS 100 μL /well and stored in the fridge overnight. After that, 200 μL of dried skimmed milk powder 3 % (w/v) and 0.05% (v/v) Tween 20 in PBS as blocking agent was added to each well of the whole plate. The plate was incubated at 37 °C for 2 h and then washed twice with 200 μL/well 0.05% (v/v) Tween in PBS and once with 200 μL/well PBS. Following that, 1.2 μL of serum from the immunised mice was added to 1 mL of PBS in 1.5ml centrifuge tubes and 100 μL/well added to the whole plate. The plate was then incubated at 37 °C for 1 h and then washed as before. Goat anti-mouse IgG1 or IgG2 labelled horse radish peroxidase was diluted with 25% (v/v) feotal calf serum in PBS at a ratio of 1:20000 and 100 μL/well added to the whole plate. The plate was incubated at 37 °C for 45 min and washed thrice with 200 μL/well 0.05% (v/v) Tween in PBS and twice with 200 μL/well PBS. The plate was dried, and 100 μL/well of TMB stabilized chromogen added to each well and left for 5 min in the darkness. The solution changed from pale yellow to blue, and then 100 μL of 10% (v/v) sulphuric acid was added to stop the reaction and then the absorbance read using a microplate reader at 450 nm.

2.9 Splenocyte stimulation with antigen

The spleen was removed from immunised mice and collected in RPMI 1640 media. The spleen was ground (with the bottom of a 2 mL cylinder) through monofilament cloth and was pipetted into a Universal tube. The cell suspension was centrifuged at 3000 g for 5 min. The supernatant was decanted and the pellet resuspended in 1 mL Boyle's solution,

vortexed and left at room temperature for 5 min. The suspension was then centrifuged at 3000 g for 5 min, the supernatant decanted and the pellet resuspended in 5 mL unsupplemented RPMI 1640 media. This step was replicated three times and the pellet resuspended in 2 mL complete RPMI 1640 media (supplemented with 10% v/v FCS, 0.1% v/v L-glutamine, 0.1% penicillin-streptomycin v/v). After that, 15 µL of trypan blue was mixed with 15 µL of the cell suspension and the cells counted on a haemocytometer. The cell suspension was diluted to 5 x 10^6 cells/ml, then, 100 µL added per well in triplicate to a precoated plate (this was carried out the day before with 100 µL of RPMI 1640 media, 10 µg/mL concanavalin A in RPMI and 0.4 µg/100mL influenza antigen in RPMI media in the different wells). After the samples were added into the plates, some were kept at 37 °C for proliferation assay (section 2.11), while the remainder were incubated at 37 °C for 72 h and kept at -20 °C until cytokine analysis was carried out (section 2.10).

2.10 Cytokine analysis

Plates were washed out thrice using 200 µL PBS with 0.05% (v/v) Tween 20 between assay steps as detailed below. Ninety-six well microplates were pre-coated the day before at 4 °C with 50 µL of 2 µg/ml purified anti-mouse IFN-ɣ antibody in PBS (pH 9). The plate was washed and the wells blocked with 150 µL of 10% (v/v) FCS in PBS (pH 9) and incubated at 37°C for 1 h. The plates were washed and 50 µL of samples (section 2.9) in triplicate wells and 30 µL of duplicate sets of standard (starting with 20 pg in 1:2 serial dilution with PBS 7.4) were added and incubated at 37 °C for 2 h. The plates were washed and 100 µL of 1 µg/ml biotin rat anti mouse IFN-ɣ antibody in 10% (v/v) FCS in PBS (pH 9) was added and incubated at 37°C for 1 h before washing. One hundred µL of AKP

Streptavidin was pipetted in a ratio of 1: 2000 (diluted in 10% v/v FCS in PBS, pH 9) and plates were incubated at 37°C for 1 h before washing. Finally, 100 µL of 1 mg/ml 4-nitrophenyl phosphate disodium salt hexahydrate in glycine buffer (7.51 g of glycine, 203 mg of magnesium chloride, 136 mg of zinc chloride in 1 L of distilled water and pH adjusted to 10.4) was added, covered with aluminium foil and incubated at room temperature for 20 min. The absorbance was then read at 405 nm. Cytokine concentrations in unknown samples was determined using the standard curve from the cytokine standard data.

2.11 Proliferation assay

Splenocytes (section 2.9) stimulated with 0.4 µg/100µL/well of influenza antigen were incubated at 37°C for 48 h. After that, 20 µL of 0.1 mg/ml resazurin was added to the samples and the plate incubated at 37 °C for 24 h. The absorbance was read at 570 nm and the percentage proliferation calculated using the equation:

$$\%\ \text{Proliferation} = \frac{\text{OD at 570nm treated(antigen)}}{\text{OD at 570nm untreated(medium)}}\ \text{x}\ 100$$

2.12 Statistical analysis

All of the experiments were analysed using Minitab® 17 software. Statistical significance was assessed by one-way analysis of variance (ANOVA) and Tukey multiple comparison test and a t-test was performed for paired comparisons. All data in graphs represent the mean and standard deviation in triplicate and a value of $p<0.05$ was considered to be statistically significant.

3 Results

3.1 Niosome characterisation

Niosomes prepared by both methods containing proteins (albumin and influenza HA antigen) were analysed by Zetasizer and the Z-average, PDI, and zeta potential values are shown in table 2. Morphological analysis using AFM showed that all the niosomes were spherical in shape with particles size comparable with the Zetasizer results (Figure 1).

Table 2 vesicle size, PDI, and ZP of vesicles prepared by different methods, containing albumin and influenza antigen.

Formulation	MM method		TFH method	
	Albumin	**Influenza HA**	**Albumin**	**Influenza HA**
Z-average (nm)	122.1 ± 0.3	157.9 ± 1.8	352.9 ± 0.9	388.8 ± 10.0
PDI	0.082 ±0.013	0.193 ± 0.014	0.266 ± 0.003	0.464 ± 0.025
Zeta potential (mV)	-18.7 ± 2.0	-14.6 ± 1.5mV	-28.4 ± 1.9	-16.6 ± 2.1

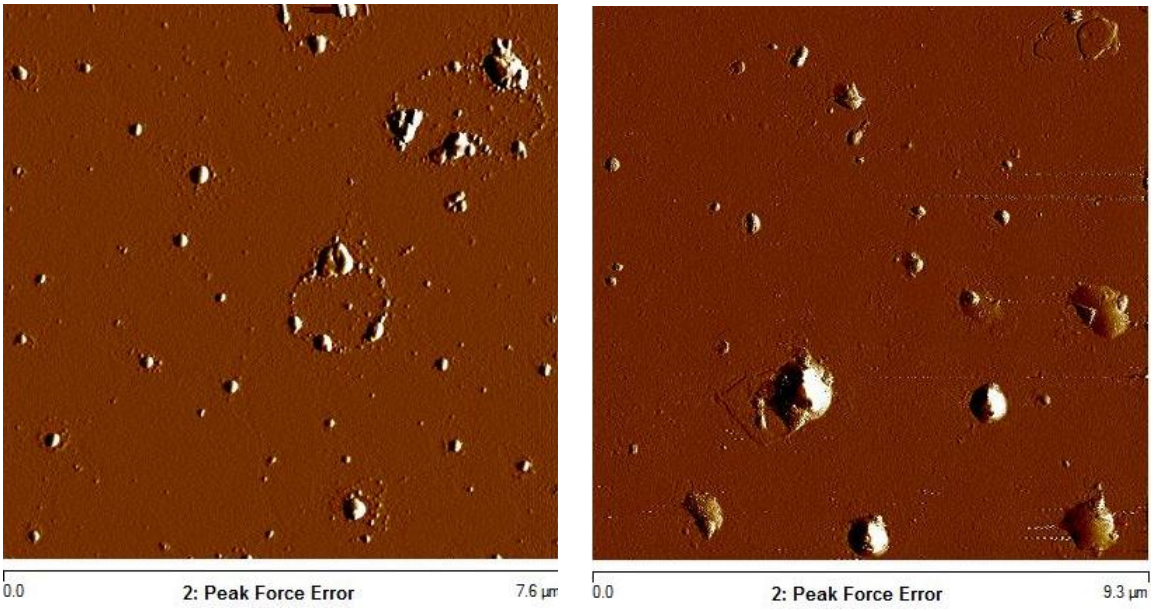


Figure 1 Micrographs of niosomes prepared by MM (left) with a 7.6 µm vertical bar and niosomes prepared by the TFH method (right) with a 9.3 µm vertical bar taken using AFM.

3.2 Entrapment efficiency

From the BCA protein assay the MM niosomes with albumin and HA antigen showed 51.63 ± 10.28% and 57.24 ± 16.06% entrapment efficiency, respectively, while the entrapment efficiency was 48.91 ± 27.35% and 47.64 ± 2.93% for the TFH method niosomes with albumin and HA antigen, respectively (Figure 2). However, there was no significant difference in entrapment efficency for the formulations (p=0.897).

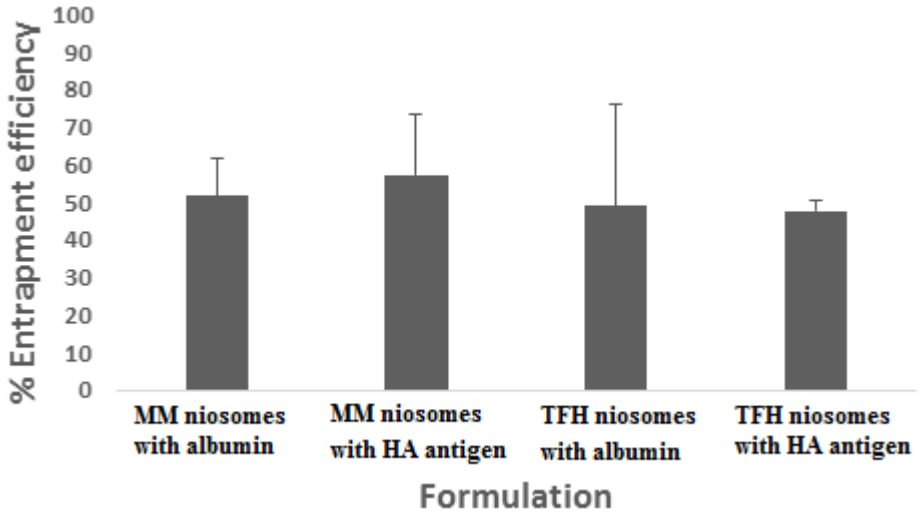


Figure 2 The protein entrapment efficiencies into niosomes prepared by MM and TFH methods. Protein content was analysed by BCA assay with no significant difference between the formulations (p=0.897). Each bar represents the mean of the percentage entrapment efficiency and standard deviation in triplicate.

3.3 IgG1 and IgG2a antibody analysis

Specific serum IgG1 (Figure 3a) was produced by all the groups after the second immunisation. Mice injected with the MM niosomes showed the lowest response, while mice injected with the control (antigen only) showed the highest. There was no significant difference in antibody titres between the TFH method niosome group and the control. After the third immunisation, the TFH method niosome and control groups showed no difference in specific IgG1 responses, but the titres induced by MM niosomes were significantly ($P < 0.05$) lower than those induced by TFH method niosomes. By the end of the study all the groups showed the same level of IgG1.

Specific IgG2a (Figure 3b) was produced by all groups after the second immunisation. Mice immunised with MM niosomes showed the lowest antibody response, while the control showed the highest. However, there was no significant difference between the groups. After the third immunisation, the TFH niosome and control groups showed no difference in specific IgG2a titres while the MM noisome group were significantly lower. By the end of the study, the MM noisome group showed the lowest specific IgG2a levels, while the control showed the highest. However, there was no significant difference between groups.

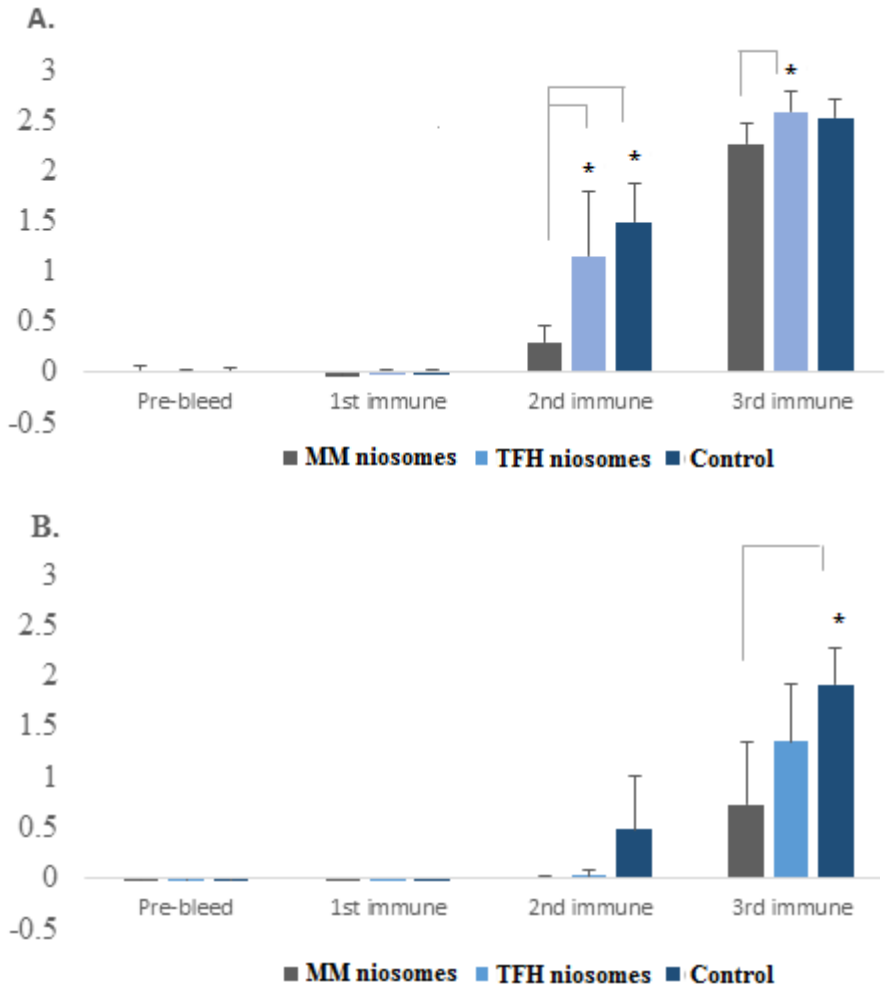


Figure 3 A. IgG1 and B. IgG2a response of mice (n=5) different treatments with entrapped *A/Switzerland/9715293/2013* influenza antigen (15 µg/0.1 ml/dose); in MM niosomes, TFH niosomes, and control (without entrapment). Each bar represents the mean of triplicate readings. *indicates significant difference ($p<0.05$).

3.4 IFN-ɣ cytokine analysis

Splenocytes collected 2 weeks after the last immunisation with influenza antigen were stimulated with influenza antigen and IFN-ɣ cytokine analysis was carried out (Figure 4). Stimulation of spleen cells from mice, immunised with antigen alone, or antigen formulated into MM niosomes or TFH niosomes, with specific antigen resulted in

significantly higher production of IFN-ɣ compared to coresponding unstimulated controls ($P < 0.05$, mean, ng/ml, ± standard deviation, 21.93 ± 16.17, unstimulated control; 78.08 ± 81.43, MM niosomes; 102.99 ± 36.84, TFH niosomes). Only, cells from mice immunised with TFH niosomes produced a significantly higher amount of of IFN-ɣ compared to cells from mice immunised with antigen alone ($P<0.05$).

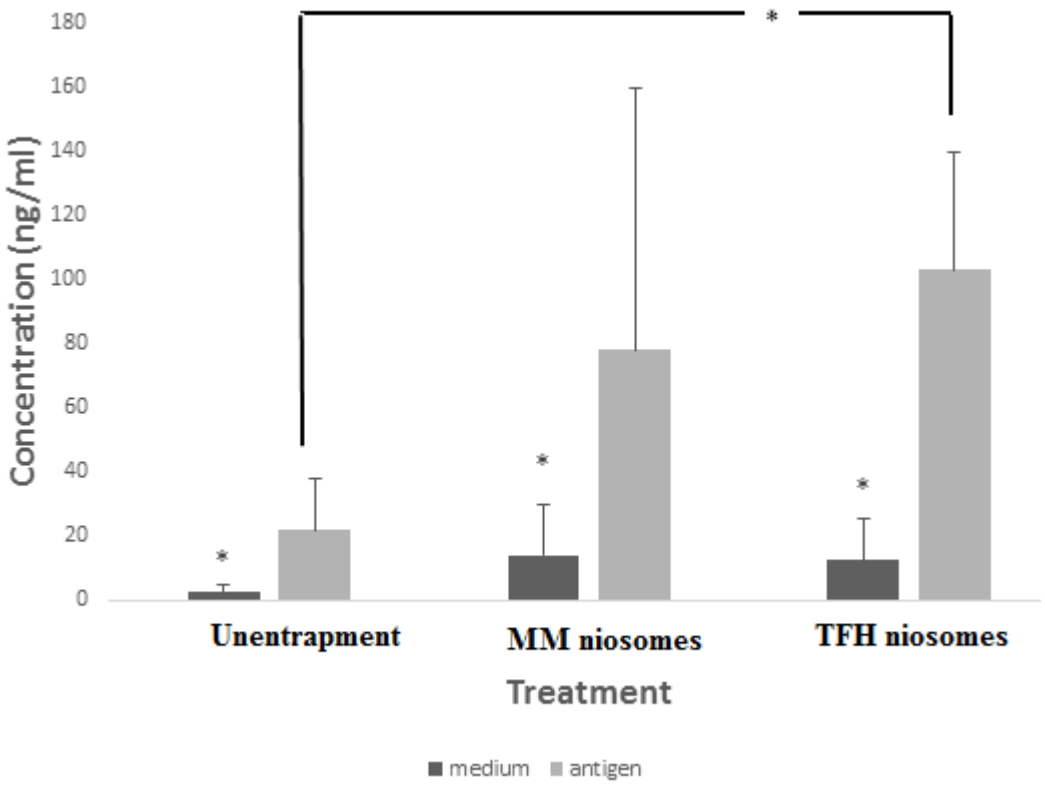


Figure 4 IFN-ɣ production from splenocytes of mice (n=5) immunised with influenza antigen and stimulated with influenza antigen. The result shows that there were significant differences in mean of concentration IFN-ɣ in each treatment compared with the negative control (no stimulation with antigen). Moreover, the IFN-ɣ produced by the TFH niosomes was significantly higher than the unentrapped group. *indicates significant difference ($p<0.05$).

3.5 Resazurin proliferation assay

Splenocytes from mice immunised above were assessed for their ability to proliferate in response to specific antigen stimulation (0.4 µg/100µL) using a resazurin proliferation assay (Figure 5). There was no significant difference (p=0.310) in the proliferative response of cells from mice immunised with MM, TFH niosomes, or antigen alone control.

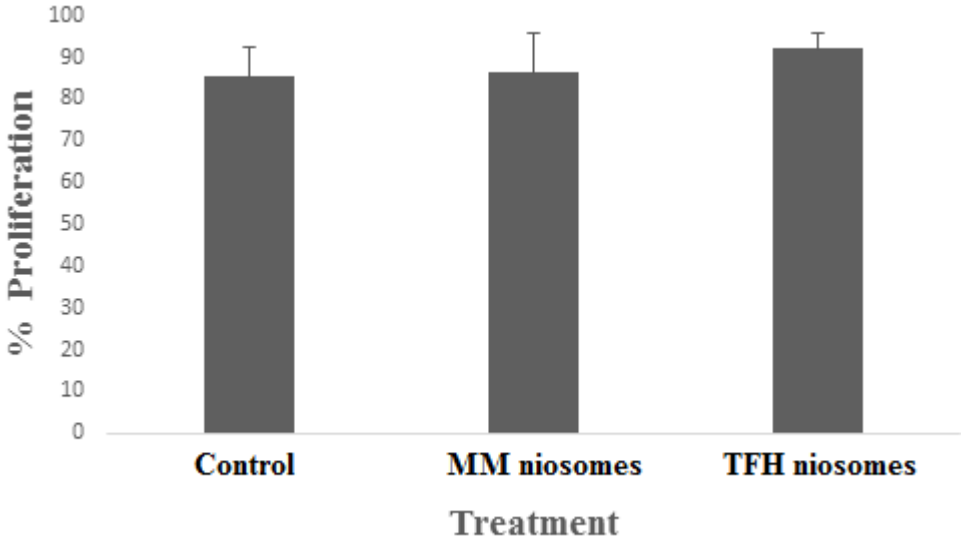


Figure 5 Influenza stimulated splenocytes analysed by resazurin proliferation assay.

4 Discussion

It has been previously reported that two different sizes of bilosomes, which are composed of niosomes and bile salt, prepared using a traditional TFH method with inactivated influenza antigen induced different systemic and mucosal immune responses (21). The result was a skewing of the Th1 and Th2 responses, depending on size. Small sized bilosomes induced a Th2 response (IgG1) whereas a mixed size of bilosomes both large

and small bilosomes induced a Th1 response by measurement of IgG2a and IFN-ɣ production. The aim of this study was to examine if different manufacturing methods of niosomes (which will end up in the preparation of niosomes with different sizes) could also cause different Th responses, particularly as using MM could control the size of the small particles produced more accurately. In this case, small niosomes were prepared by MM and large niosomes were prepared by the traditional TFH method and using a mini-extruder to produce a more uniform size distribution.

Albumin was used as a model antigen in the preliminary studies to optimize the niosome formulations and then repeated with influenza HA antigen (22). Both types of vesicle (MM and TFH niosomes) with influenza antigen were larger than vesicles with albumin. This is most likely due to the fact that the molecular weight of albumin (44.3 kDa) (23) is lower than influenza antigen (61.8 kDa) (24). A study by Vangala *et. al.* confirms that vesicle diameters increase significantly due to the molecular weight of the antigen (25).

In addition, the PDI of the MM niosomes, which was narrow by twice the dispersion compared with the TFH niosomes because of the precision of the machine used in preparing the MM niosomes. Zidan *et al.* (2011) also found that the PDI of niosomes prepared by MM showed significantly ($p < 0.05$) decreased polydispersity compared with niosomes prepared by TFH method (26, 27). These findings were comparable with the results in this work where the MM method produced small and uniform particles.

In terms of Th1/Th2 response in relation to the different sizes of the niosomes, the results showed that encapsulated influenza antigen in MM niosomes (158 nm) and TFH niosomes (388 nm) induced both IgG1 and IgG2a. In addition, the level of IFN-ɣ

production after the administration of the TFH niosomes was significantly ($p < 0.05$) higher compared to unentrapped formulation, while the MM niosomes showed no significant difference. During the immunisation stages, there was differences in the IgG1 and IgG2a levels between the mice that were immunised with influenza antigen encapsulated MM and TFH niosomes. A previous study demonstrated that niosomes with a mean diameter of more than 225 nm induced a Th1 response by production of IgG2a, whereas smaller sized niosomes with a mean diameter less than 155 nm induced a Th2 response by producing IgG1 without IgG2a after subcutaneous administration (17). This partially agreed with the results of the present study in that the TFH niosomes with a mean diameter of 388 nm induced both IgG1 and IgG2a antibodies, however the preference to induce a Th1 response was indicated due to the fact that the production of IFN-ɣ increased significantly compared with unentrapped formulation. Macatonia *et al.* confirmed the induction of a Th1 response that was dependent on production of IFN-ɣ (28). In addition, the Mann *et al.* (2009) study also agreed that large bilosomes (980 nm) induced a Th1 response as measured by IgG2a and IFN-ɣ production (29). On the other hand, smaller niosomes with a mean diameter of 388 nm induced both IgG1 and IgG2a. So, from these results, it is clear that the response toward immunisation using an adjuvant is dependent on the particle size of the delivery system which were prepared by different manufacturing methods.

The resazurin proliferation assay showed that the spleen cells from animals treated with TFH niosomes, Mm niosomes, and unentrapped formulation were not significantly different, although the proliferation rate with TFH niosomes was highest (91.94%),

compared with the MM niosomes (86.35%) and the unentrapped antigen (85.28%) formulation. The proliferation assay is one method to confirm the T-cell response to specific antigen (30). This proliferation of lymphocyte response to antigen occurs when the animals were immunised against the antigen. When the antigen enters the cells, it is presented via MHC class I and II which release antigen-specific antibodies, including activation and proliferation of antigen-specific cytotoxic T lymphocytes for elimination of the antigen. However, T-cells remain to memorise, prevent production of viral progeny (31) and protect against the same antigen (32). As a result, a high rate of proliferation displays a high response. With reference to the present study, stimulation of cells from animals treated with TFH and MM niosomes were slightly more responsive than the control, and therefore such niosomes are likely to act as an adjuvant to help produce a better response against the vaccine (33).

Conclusion

Niosomes prepared using the TFH technique with a mini-extruder produced large sized vesicles with a broad distribution. However, the MM technology produced niosomes of smaller size, with more uniformity and narrower distribution. The encapsulated influenza antigen of MM niosomes (158 nm) and TFH niosomes (388 nm) induced both IgG1 and IgG2a. Both vesicles showed a Th2 response based on IgG1 production with no significant difference by the end of the study, whereas the early part of the study showed that TFH niosomes induced a Th2 response equivalent to the current vaccine. However, TFH niosomes showed a Th1 response based on IFN-ɣ production with significant

difference compared with the current vaccine, but MM niosomes showed no significant difference. This experiment indicated that TFH niosomes induces a Th1 response, whereas MM niosomes induce a Th2 response.

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