Title: Characterization and Optimization of Bilosomes for Oral Vaccine Delivery

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Abstract

Oral vaccines offer significant benefits due to the ease of administration, better patient compliance and non-invasive, needle-free administration. However this route is marred by the harsh gastro intestinal environment which is detrimental to many vaccine formats. To address this, a range of delivery systems have been considered including bilosomes; these are bilayer vesicles constructed from non-ionic surfactants combined with the inclusion of bile salts which can stabilise the vesicles in the gastro intestinal tract by preventing membrane destabilisation. The aim of this study was to investigate the effect of formulation parameters on bilosome carriers using Design of Experiments to select an appropriate formulation to assess *in vivo*. Bilosomes were constructed from monopalmitoyl glycerol, cholesterol, dicetyl phosphate and sodium deoxycholate at different blends ratios. The optimised bilosome formulation was identified and the potential of this formulation as an oral vaccine delivery system were assessed in biodistribution and vaccine efficacy studies. Results showed that the larger bilosomes vesicles (~6 μm versus 2 μm in diameter) increased uptake within the Peyer's patches and were able to reduce median temperature differential change and promote a reduction in viral cell load in an influenza challenge study.

Introduction

An effective vaccine is one which prevents infection and disease through its capability to elicit specific immune responses and hence provide protective immunity (Bramwell and Perrie, 2005). However ease of use is also a key consideration for global vaccination campaigns. In this respect, oral vaccines offer significant benefits over traditional vaccines due to their ease of administration, improved patient compliance (due to their needle-free format), potentially enhanced mucosal immunity and strong resistance against many pathogens. Furthermore due to its non-invasive nature and ease of administration, the oral route can circumvent the need for trained personnel to administer the vaccine. However, the ability of many vaccines to withstand the harsh journey through the gastrointestinal tract, and to be effectively taken up in the appropriate target site is limited (Wilkhu et al, 2011).

Therefore to circumvent this barrier, delivery systems can be employed. For example, lipid based vesicles such as liposomes or niosomes (non-ionic surfactant vesicles) can be used as effective carriers of macromolecules (Bramwell and Perrie, 2006). Yet, many of these constructs can be destabilised after exposure to intestinal bile salts, which have the potential to cause the membrane deformation and vesicle lysis, resulting in the release of macromolecules from the vesicle prior to reaching its intended site of action. In response to this, a range of modified lipid based vesicles, which have increased stability and increased absorption through the GIT, have been developed. Such vesicles include bilosomes, which have been shown to protect antigens from the enzymes present in the GIT and act as potent immunological adjuvants (Mann et al., 2009, Shukla et al., 2008, Mann et al., 2004). This has been attributed to the incorporation of bile salts into the vesicle bilayers, which increases resistance to degradation and disruption by digestive enzymes (Schubert et al., 1983).

For effective delivery of vaccines after oral administration, the target site for vaccines is the M cells located in the Peyer's patches, which are responsible for secretory IgA and other mucosal responses (Schmucker et al., 1996). As a result of bilosomes ability to protect and target antigens, smaller concentrations of the antigen can be used to provide an effective response relative to unformulated antigen given orally. This has been demonstrated by e.g. Conacher *et al*, 2001 who showed that including bile salts in the vesicle constructs had a stabilising role after oral administration by preventing degradation with highly acidic conditions and improving oral vaccine efficacy (Norris et al., 1998, Conacher et al., 2001). Similar studies have demonstrated this with a range of antigens e.g. tetanus toxoid (Mann et al., 2006), the A/panama (Mann et al., 2004), Diphtheria toxoid (Shukla et al., 2011) and hepatitis B (Shukla et al., 2010). These studies all show that, by orally administering the bilosome vesicles with entrapped antigen, a mucosal immune response is elicited with specific IgA production increased. Therefore, bilosomes can aid vaccine technology by increasing the stability of the vaccine during transit through the GIT, preventing premature release and/or degradation of the antigen during oral transit (Wilkhu et al., 2011).

From these studies several potential controlling parameters for the design of bilosome constructs become apparent: vesicle size is vital as this determines the end location of the vaccine when administered, the zeta potential and the vesicle pH is also important when determining the stability and absorption of the vaccine (Norris et al., 1998). Therefore within this study we have undertaken a systematic investigation into the effects of bilosome composition on their physico-chemical characteristics and the relationship between the factors influencing the bilosome composition and physiological and biological outcomes.

Design of Experiments, using a d-optimal factorial design, was used to plan and conduct experiments in a random order and used to extract information on the relationship between formulation and function.

Materials and methods

Materials

To form the vesicles the lipids monopalmitoyl glycerol (MPG; Larodan AG, Sweden), synthetic cholesterol (Chol), dicetyl phosphate (DCP) and sodium deoxycholate (bile salt) (Sigma-Aldrich, UK) were used. The buffers were made up of sodium bicarbonate (Sigma-Aldrich, UK) at two specific pH, where hydrochloric acid and sodium hydroxide (NaOH) (Sigma-Aldrich, UK) was used for pH adjustments. For the antigen a recombinant HA or the H3N2 sub-unit protein (Immune Tech, USA) was used.

Preparation of bilosomes.

Bilosomes were prepared based on a modified method of Conacher et al, (Conacher et al., 2001). Briefly, a hot paraffin oil bath was set up at 120 °C and alongside a water bath at 50 °C. Two 25 mM sodium bicarbonate buffers were made up, one at a pH of 7.6 and the other with a pH of 9.7. The bile salt solution was prepared by making a 100 mM solution using the freshly made 25 mM sodium bicarbonate buffer of pH 9.7. Appropriate molar ratio of the lipids MPG, Chol and DCP were weighed and placed in a 25 mL flat bottom glass beaker ensuring no powder sticks to the sides of the glass beaker. The lipids were melted by heating at 120 °C for 10 minutes with occasional swirling.

While maintaining the molten lipid (308.5mg) at 120 °C, an emulsion was created by the addition of 5.2 mL of the 25 mM sodium bicarbonate pH 7.6 buffer (preheated to 50 °C) and immediately homogenised (using an emulsion head) for 2 minutes at 8000 rpm. While still homogenising 0.55 mL of 100 mM bile salt in 25 mM sodium bicarbonate buffer pH 9.7 was added and homogenised further for 3 minutes. After the 3 minutes homogenising, 0.25 mL (10 mg/mL) of the pre-heated (50 °C) antigen solution was added to the beaker containing the suspension, and homogenised for a further 5 minutes. The antigen was added at the final stage to minimise exposure of the antigen to homogenisation. Once homogenisation had finished, the bilosome suspension was allowed to cool to 30 °C, and left for 2 hours in an incubator/shaker at 220 rpm.

Characterization of vesicles.

The size of the bilosomes was determined using laser diffraction on a sympatec 2005 (Helos/BF) analyser. 20 µL of the bilosome suspension was diluted into the cuvette with 40 mL double distilled water. The zeta potential, which is an indirect measurement of the vesicle surface charge, was measured in 1.5 mL double distilled water at 25 °C on a Zeta Plus Brookhaven Instrument. 20 µL of the bilosome suspension was mixed in 1.5 mL double distilled water and then analysed. The pH of the vesicle suspension was determined using a pH meter where the tip was placed into the bilosome suspension and left for a few minutes.

Radiolabelling of H3N2 antigen

To further quantify antigen incorporation, the antigen was radio-labelled using ^{125}I. To achieve this antigen stock solutions were prepared as 100 µg (1mg/mL) in PBS stock and 40 µL (40 µg) placed into an iodination tube (Pierce Biotechnology) with 2 Mbq for 1 hour and

subsequently separated from non-labelled antigen by column chromatography (Henriksen-Lacey et al., 2010).

Stability of the vesicles in simulated fasted gastric and intestinal medium

Fasted state simulated gastric media (50 mL) was prepared using a 34.2 mM NaCl solution in 50 mL HPLC water at pH 1.2 adjusted with 1 M HCl. Pepsin (40 mg) was then added, followed by sodium taurocholate (2.15 mg) and phosphatidylcholine (0.76 mg) in 50 mL at 37 °C (Vertzoni et al., 2005).

Fasted state simulated intestinal media (50 mL) was prepared by a 50 mM PBS solution in 50 mL HPLC water at pH 8.5, adjusted with 1 M NaOH, and sodium glycodeoxycholate (180 mg) and phosphatidylcholine (34 mg) dissolved in the solution at 37 °C. To establish the effect of these conditions on vesicle attributes, 400 μL of the vesicle formulations was added to 3.6 mL of Fasted gastric medium as a 1:10 dilution. The formulations were tested for vesicle size and zeta potential at specific time intervals. The gastric to intestinal phase was carried out by centrifuging 3.9 mL of the formulations from the gastric period and then resuspending the pellet in fasted intestinal fluid and was then tested at the stated time points. To measure antigen retention in these conditions, radio-labelled antigen was incorporated within the formulations and antigen retention tracked in the above conditions by ultracentrifugation (Beckaman, Ultima XP) at 100,000g.

***In vivo* biodistribution protocol**

Inbred female Balb/c (6-10 weeks of age) mice were housed in cages within a laminar flow safety enclosure and provided with irradiated food and filtered drinking water. Experimentation adhered to the 1986 Scientific Procedures Act (UK). All protocols have been subject to ethical review and were carried out in a designated establishment. 200 μL

doses of the formulations, which were washed to remove unentrapped radiolabelled antigen, were given orally to Balb/c mice in groups of 4. Animals were terminated at various time points, organs collected and analysed for both ^{125}I (to measure antigen) and ^{3}H, which was used as a radio-active tracker for vesicles by incorporating ^{3}H-cholesterol in the formulation.

Gamma vials were pre-labelled and individual tissues/organs were weighed and individually placed into the gamma vials. To the gamma vials 1.5 mL of solvable was added to digest the tissues. Once the solvable was added to the vials they were then placed onto the gamma counter to record the ^{125}I-antigen levels. The vials were then placed into an incubator at 50 °C overnight to dissolve the tissues. Once the tissues had dissolved the contents of the gamma vials were transferred to 20 mL scintillation vials where 200 µL hydrogen peroxide was added to each vial to bleach the samples. The vials were left overnight once again to wait for gas to disappear and 10 mL of scintillation fluid (Ultima Gold) was added to form an emulsion. The vials were then counted on the scintillation counter which will represent the counts for the vesicles at each site.

Oral Vaccine study

Bilosome vesicles were prepared with recombinant HA directed against influenza viruses as per protocol and were orally administered to ferrets on days 0, 3, 14, and 17, and 14 days later challenged with a clinical rHA isolate of influenza A. As the key correlate of protection, median temperature differential was measured to follow protection against fever and inflammatory cell counts in nasal washes were measured.

Statistical analysis

The results within this study are given as the geometric mean ± S.D. unless stated otherwise. The DOE statistics were carried out using ANOVA based within the software MODDE (Umetrics). Differences between the biodistribution antigen levels were analysed by

ANOVA. A probability factor of less than 0.05 ($P < 0.05$) was considered to represent statistically significant difference.

Results and Discussion

Design of experiments response

An experimental design consisting of MPG, Cholesterol, DCP and bile salt as the factors was generated with the responses tested being pH, vesicle size and zeta potential. MPG was always kept constant, at a molar ratio of 5, whereas the cholesterol, DCP and bile salt were varied. The experimental design consisted of three centre points to form the control, by appearing in a random order, with the same molar ratios for each of the factors. The centre points are calculated by taking the average of each factor for all the experimental runs and in turn will determine the accuracy and reproducibility of the experimental model that is calculated.

The partial least squares (PLS) orthogonal coefficients of the bilosome composition on pH, zeta potential and vesicle size are shown in Fig 1A to C, respectively; these results show three significant variables which are highlighted from the results of the d-optimal factorial design that involve cholesterol, DCP and bile salt. In particular, the DCP and cholesterol content together are shown to have a significant impact on the pH and zeta potential (Fig 1A and B) with the *P*-value being less than 0.05 (Table 1). However, in terms of vesicle size, statistically the cholesterol and DCP ratio made no impact (Fig 1C; Table 1). Interestingly, only vesicle size was shown to be influenced by bile acid content; Figure 1C shows that bile salt has a negative impact, with an increase in bile salt content to the formulation reducing vesicle size.

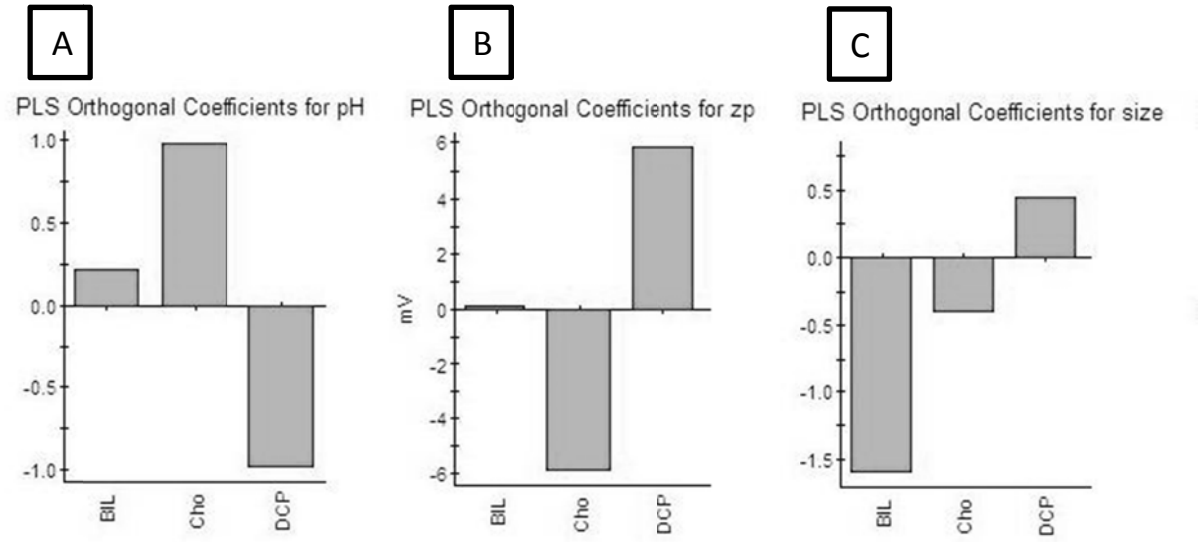

Figure 1- The partial least squares (PLS) orthogonal coefficients of the bilosome composition on (a) pH, (b) Zeta potential, (c) vesicle size. The PLS plot takes into consideration several responses and fits a model showing the variation of the responses to the factors.

Table 1 ANOVA results for pH, Zeta potential and vesicle size as a response (Y1).

Response	*F*-value	*P*-value
Suspension pH	14.8585	**0.001**
Zeta potential	10.7774	**0.002**
Vesicle size	1.53747	**0.292**

Further consideration of the impact of cholesterol/DCP ratio is shown as response surface and contour plots of DCP against cholesterol content in terms of pH and zeta potential; Figure 2 illustrates that the cholesterol and DCP content has a significant impact upon the pH of the vesicle suspension, which ranged from 3.5 to a maximum of 8.5 depending on the cholesterol/DCP content (Fig 2A and B). The zeta potential of the formed vesicles was also shown to change from -38 mV to -64 mV as the cholesterol/DCP ratio increase (Fig 2C and D). This result was counter intuitive; given that DCP is an anionic surfactant, it would be expected that increasing its content would increase the negativity of the vesicles. However

given that pH can directly impact on the zeta potential of suspended particles, the link between these two factors was further investigated (Fig 3A).

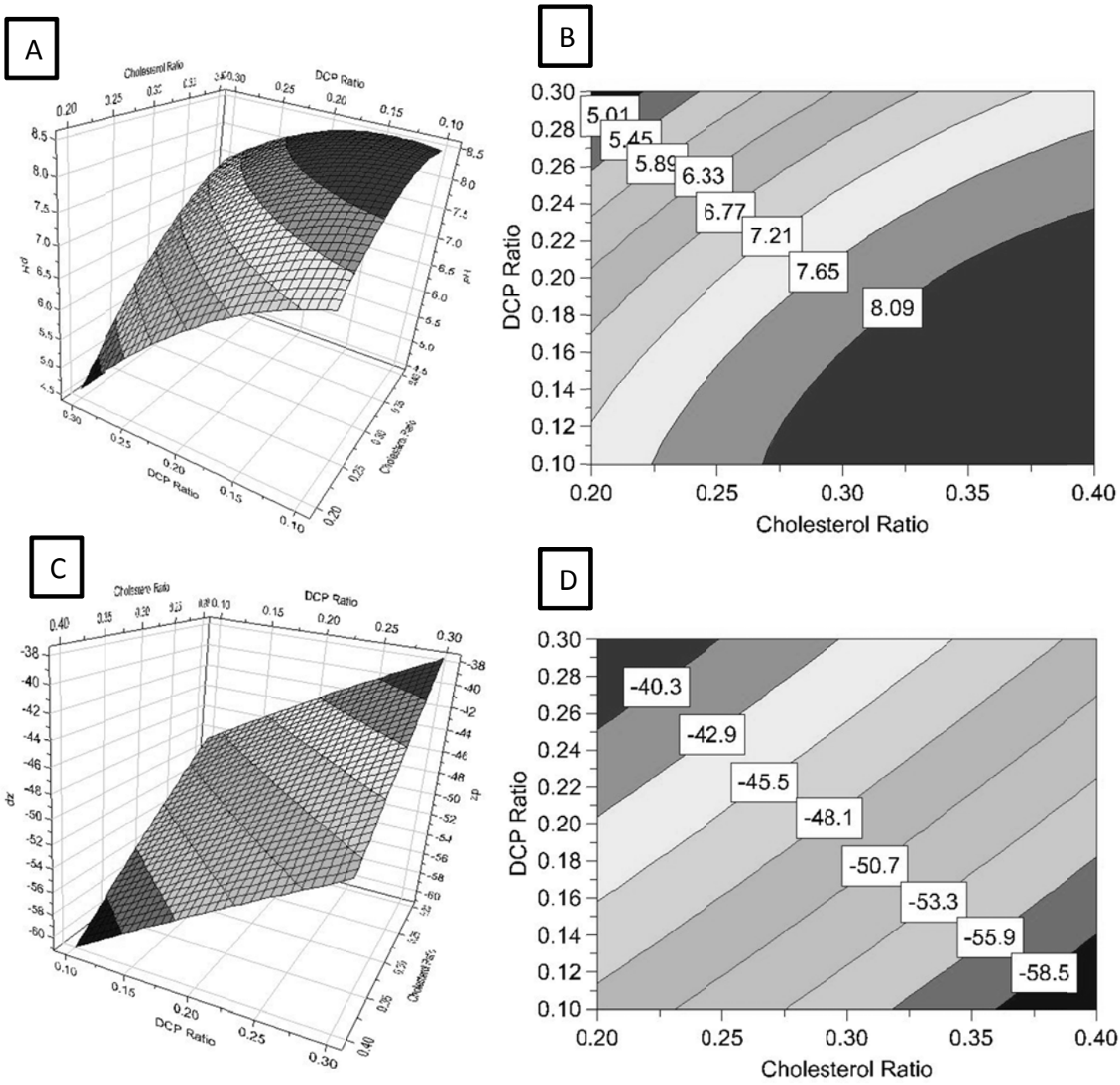

Fig 2 (a) Response surface plot showing the effect of DCP and cholesterol content on the pH, (b) Contour plot of DCP and cholesterol effects on the pH, (C) Response surface plot showing the effect of DCP and cholesterol content on the Zeta potential, (D) Contour plot of DCP and cholesterol effects on the Zeta Potential. All response plots are taken where bile salt content is at 100mM as bile salt content does not influence pH or Zeta potential.

In this study only DCP content was varied, the MPG and cholesterol ratios were kept constant at a ratio of 5:4 respectively and the bile salt content at 100 mM. Results in figure 3 show that DCP is the key factor controlling both the pH of the vesicle suspension and vesicle zeta potential, with increasing DCP content decreasing pH and reducing the negativity of bilosome zeta potential. This may be an outcome of the increasing DCP content, through its weakly acidic action, and dissociation of the $^{+}$H ion reduces the pH of the suspension, thus as the DCP content increases, the pH drops, and this could, in turn, reduce the zeta potential. The impact of DCP on vesicle suspension pH has also been studied by (Kogure et al., 1997) and Manosroi et al, 2003. Manosroi et al, (2003) were developing a liposome formulation for tranexamic acid (TA) with various lipid compositions including negatively charged DCP lipid and a positively charged stearylamine (Manosroi et al., 2003). One of the features of the study showed that negatively charged lipids (DCP) gave low pH values ranging from 3.4-7.1 compared to the positively charged lipids (pH 7.6-7.9).

To confirm this, the zeta potential of a fixed (5:4:1 MPG:Chol:DCP with 100 mM bile salt) bilosome formulation was investigated over a pH range from 1 to 10 and reversed again (Fig 3B) and the results show that as we increase the pH from 1 to 10, the zeta potential of the vesicles changes from around -15 mV to -120 mV confirming the controlling role of pH on the anionic nature of the bilosomes (Fig 3B). Therefore by varying the DCP content in the bilosome formulations, this modulates the pH of the suspension system which in turn impacts on the zeta potential of the bilosomes, thereby confirming that a continued increase in anionic surfactant content may not always promote increasing anionic zeta potential of vesicles.

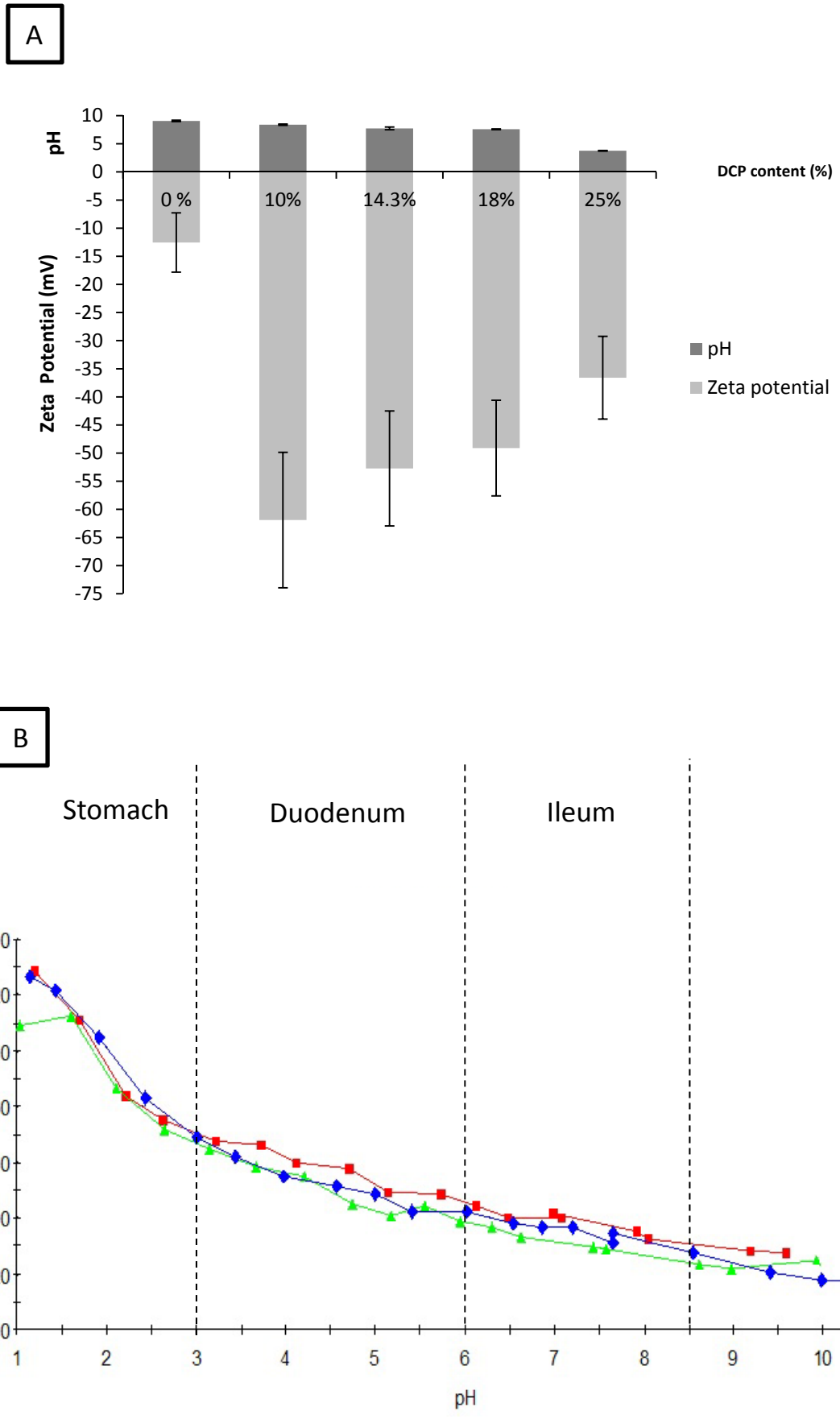

Figure 3 (a) Effect of changing DCP content on the pH and Zeta potential of the Bilosomes. (b) Effect of titrating acid on the pH and Zeta potential of the Bilosomes

In terms of how this impacts on biological responses, the zeta potential has been shown to influence the uptake of bilosomes in studies by Tomizawa *et al*. (1993) who have shown that negatively charged vesicles are preferably taken up by the Peyer's patches (Tomizawa et al., 1993). Therefore, by incorporating appropriate levels of DCP into the formulation, it gives the bilosomes their negative charge, which can be retained throughout the various pH ranges of the GIT (Fig 3B) suggesting that this will aid in the uptake of the bilosomes by the Peyer's patches at the range of pH seen across the GIT.

Effect of bile salt concentration on the vesicle size

Given that figure 1C identified bile salt concentration as a key formulation parameter, this was further investigated in figure 4, particularly given that vesicle size range may be crucial in determining uptake of particles by Peyer's patches; studies by Eldridge *et al*. (1990) on vesicle size uptake in the Peyer's patches show that uptake is dependent on the vesicle size and hydrophobicity (Eldridge et al., 1990). The results from their experiments indicate that a vesicle size of 10 μm or less are taken up by the Peyer's patches, however particles with a size less than 4- 6 μm can traffic to other tissue cells by a process of lymphoid drainage. As a result, particles larger than 4-6 μm reside within the Peyer's patches, the vesicles formulated in this study fall within this size range.

Figure 4 shows the volume mean distribution (VMD) of vesicles for each formulation, with all formulations in this study were made with a 5:4:1 ratio of lipids (MPG: Chol: DCP) with the bile salt concentration ranging from 0 to 800 mM. Results showing that as the bile salt concentration increases the vesicle size decreases, whist the size distribution was not significantly influenced. A previous study incorporating bile salt within lipid vesicles by Chen et al., (2009) has shown the same trend of decreasing vesicle size upon increased bile

salt concentration, with this being attributed to enhanced flexibility and a lowering of surface tension between the vesicles (Chen et al., 2009). However across the bile salt concentration range tested, the formulated bilosomes were generally in the 4-6 µm particle size range therefore should reside within the Peyer's patches as required for vaccine delivery (Fig 4C and D).

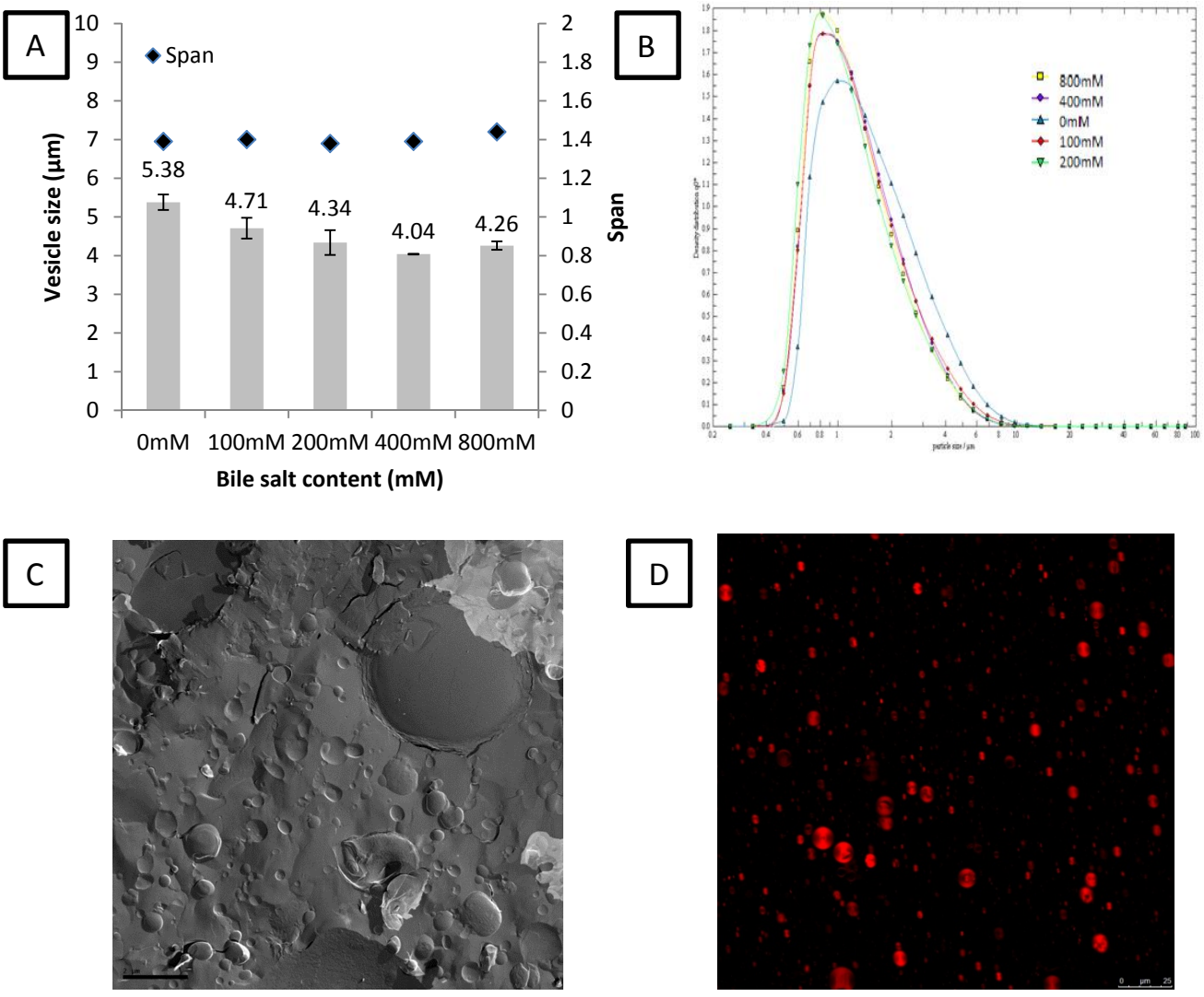

Figure 4 (a) Effect of bile salt content on mean vesicle size distribution (VMD) with span, (b) Number frequency of the formulations with increasing bile salt content in relation to the VMD. (C and D) Freeze fracture and Confocal laser microscopy images showing population of vesicles within suspension.

Bilosome stability in simulated fasted gastric and intestinal conditions.

In addition to the role of bile salt in controlling vesicle size, the primary reason for inclusion of bile salts was to investigate their potential to improve vesicle stability in the GI environment. Therefore, the characteristics of the bilosomes formulation (5:4:1 MPG:Chol:DCP with 100 mM bile salt) were tracked in simulated gastric and intestinal conditions. Table 2 shows that the bilosome vesicle size increases from 6.44 ± 0.05 μm to 11.9 ± 0.57 μm after exposure for 15 min in simulated gastric fluid but did not significantly increase in size thereafter (Table 2). The drop in pH in the simulated stomach conditions resulted in a reduction in zeta potential from -125 mV to -47 mV (Table 2). However when these bilosomes were transferred from the simulated gastric to the simulated intestinal environment, the bilosomes decreased back to their original size, and moved to a more negative, but not fully restored, zeta potential (Table 2). With regard to antigen incorporation, initial entrapment (based on initial amount added) shows that the bilosome formulation has an initial antigen loading of 32 %. When placed into the gastric media, no antigen was lost with the bilosomes maintain their antigen loading. However, when taken from gastric to intestinal medium, approximately 24 % of the antigen loading was lost, with antigen loading decreasing to 7.8-8.5 % (Table 2).

The sharp decline in antigen loading with the bilosome formulation (Table 2) when taken from gastric to intestinal fluid at the same conditions could be an indication of the special location of antigen within the vesicles. In their manufacture, the antigen is incorporated via homogenisation of formed bilosomes, and therefore incorporation may be predominately associated with the outer regions of the vesicles or surface bound. Therefore, when the vesicles move from the gastric to intestinal conditions, degradation of antigen located on the surface may occur.

Table 2: *In Vitro*- Antigen retention studies accompanied by corresponding size and zeta potential at the specific time points for each media condition.

Time (Min)		Vesicle Size (µm)	Zeta Potential (mV)	Antigen incorporation (% of initial antigen added)
0	Gastric media	6.44 ± 0.5	-125.0 ± 21	32.3 ± 1.2
15		11.50 ± 0.5	-47.0 ± 6.7	36.0 ± 1.9
30		11.31 ± 0.3	-45.4 ± 6.2	36.3 ± 0.9
60		11.90 ± 0.6	-47.0 ± 8.6	36.2 ± 1.7
75	Intestinal media	6.11 ± 0.6	-89.1 ± 21	8.7 ± 0.1
90		5.36 ± 0.3	-89.4 ± 24	8.6 ± 0.1
120		6.43 ± 0.5	-82.4 ± 10	9.2 ± 0.1
300		5.14 ± 0.2	-85.1 ± 9.5	9.3 ± 0.1

The delivery potential of bilosomes after oral administration.

Using the optimised bilosome formulation, the ability of these vesicles to carry the antigen through the GIT and deliver it to the Peyer's patch was investigated by tracking ^{125}I-labelled antigen and ^{3}H-labelled bilosomes. Mice were dosed with the various formulations via oral gavage and Fig 5 outlines the percentage of antigen with bilosomes, at time intervals of 30 minutes, 1 hour and 4 hours in various regions of the GIT. In addition antigen, administered without vesicles, was measured at 1 hour to allow comparison (given its rapid clearance no other time points for antigen alone were considered).

Figure 5A shows that after 30 min around 8 % of the vesicles can be found in the stomach and around 40 % found in the small intestine. With increasing time, the bilosomes can be seen to move from the small intestine into the cecum and colon (Fig 5A). With regard to the associated antigen, a similar trend is seen (Fig 5B), with the highest levels in the small intestine being measured after 30 min and 1 hour. However when antigen is delivered without a carrier, only low levels are measured across the GIT, with a total of 39 % being measured in the GIT compared to 55 % for antigen delivered with the bilosome carrier at the same time

point (Fig 5B), suggesting the free antigen is degraded and clearly quickly from the GIT. In particular, it is notable that higher levels of antigen were measured in the small intestine when incorporated into the bilosome carrier system.

In terms of antigen targeting to the site of action, figure 5C shows antigen recovery at the Peyer's patches and mesenteric lymph tissue for the 'free antigen' compared to the antigen associated with bilosomes vesicles. Results show that by using the bilosome carrier, a significantly higher ($p<0.05$) level of antigen was measured within the mesenteric lymph tissue when delivered using the bilosome carrier compared to antigen given alone without a carrier system. Interestingly whilst higher levels of bilosomes were measured in the Peyer's patches compared to MSN, this did not translate to increased antigen levels, with comparable bilosome delivered antigen being measured in the Peyer's patches and the MSN. This could be indicative of different trafficking of the antigen and bilosomes, potentially with the dose of surfactant having an impact on the amount of bilosomes moving to the MSN.

The impact of vesicle size on oral distribution

To investigate the impact of vesicle size, bilosomes were reduced in size via probe sonication to 1.88 μm and the distribution study repeated (Fig 6) using 30 min as the end point based on the results from figure 5. The antigen and vesicle recovery data suggests that the majority of the formulation after a 30 minute is present within the small intestine of mice, with approximately 10 % in the stomach (Fig 6). These results are in line with results for the larger (6.44 μm) vesicles (Fig 5A).

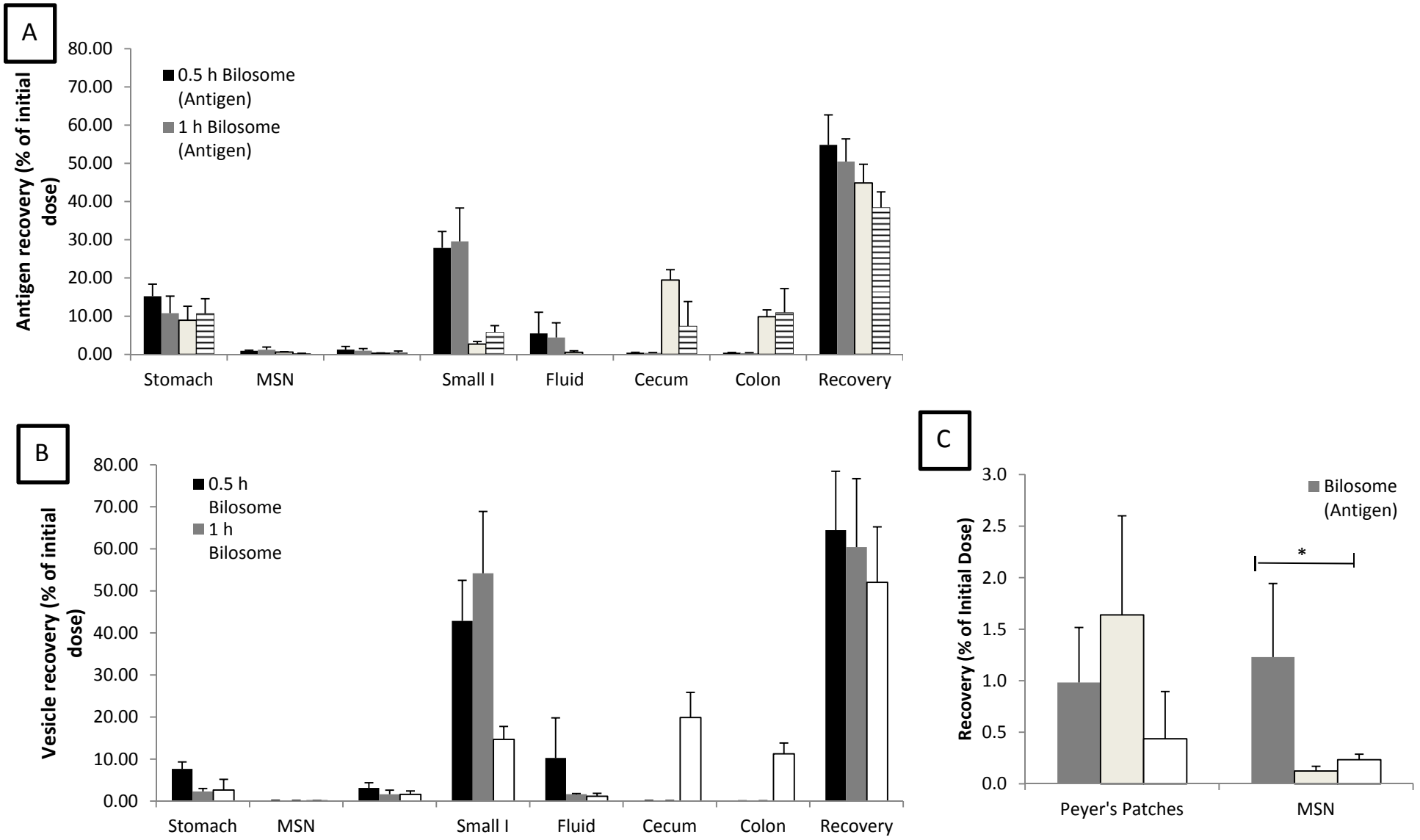

Figure 5. (A) vesicle recovery within organs after dosing regime including free antigen, (B) corresponding antigen recovery within the organs and (C) closer look at the Peyer's patch uptake and Mesenteric lymph tissue recovery of antigen and vesicle after 1 h timepoint (*significant difference $p < 0.05$).

Considering the target site uptake of antigen and carrier into the Peyer's patch and mesenteric lymph tissue (Fig 6B), significantly higher ($p<0.01$) levels of the larger vesicles was found in the Peyer's patches compared to the smaller vesicle size formulation (Fig 6B). However this did not translate into significantly increase the levels of bilosomes or antigen in the MSN. A similar trend has been observed by Ebel (1990) who compared the uptake of 9 μm and 2 μm polystyrene latex beads via the Peyer's patches and Mesenteric lymph nodes. The results showed that the larger particles retained within the Peyer's patches with no presence in the mesenteric lymph nodes, whereas the smaller particles were more noticeable in the mesenteric lymph nodes (Ebel, 1990). Further, in studies using PLA, Eldridge et al., (1990) demonstrated that Peyer's patch uptake was restricted to particles less than 10 μm, however whilst particles between 5 and 10 μm remained within the Peyer's patches, the majority of microspheres less than 5 μm were transported through the efferent lymphatics within macrophages, and suggest that this pattern of absorption and redistribution may determine the type of immune response elicited by the vaccines, with microspheres below 5 μm inducing a predominantly circulating antibody response, while those above this size would stimulate a mucosal (IgA) immune response (Eldridge et al., 1990). Therefore, the results in figures 5 and 6 demonstrate that larger (~ 6 μm VMD) vesicles promote enhanced uptake and retention of both vesicles and antigen within the Peyer's patch and mesentery lymph nodes potentially offering a greater chance to increase mucosal immunity.

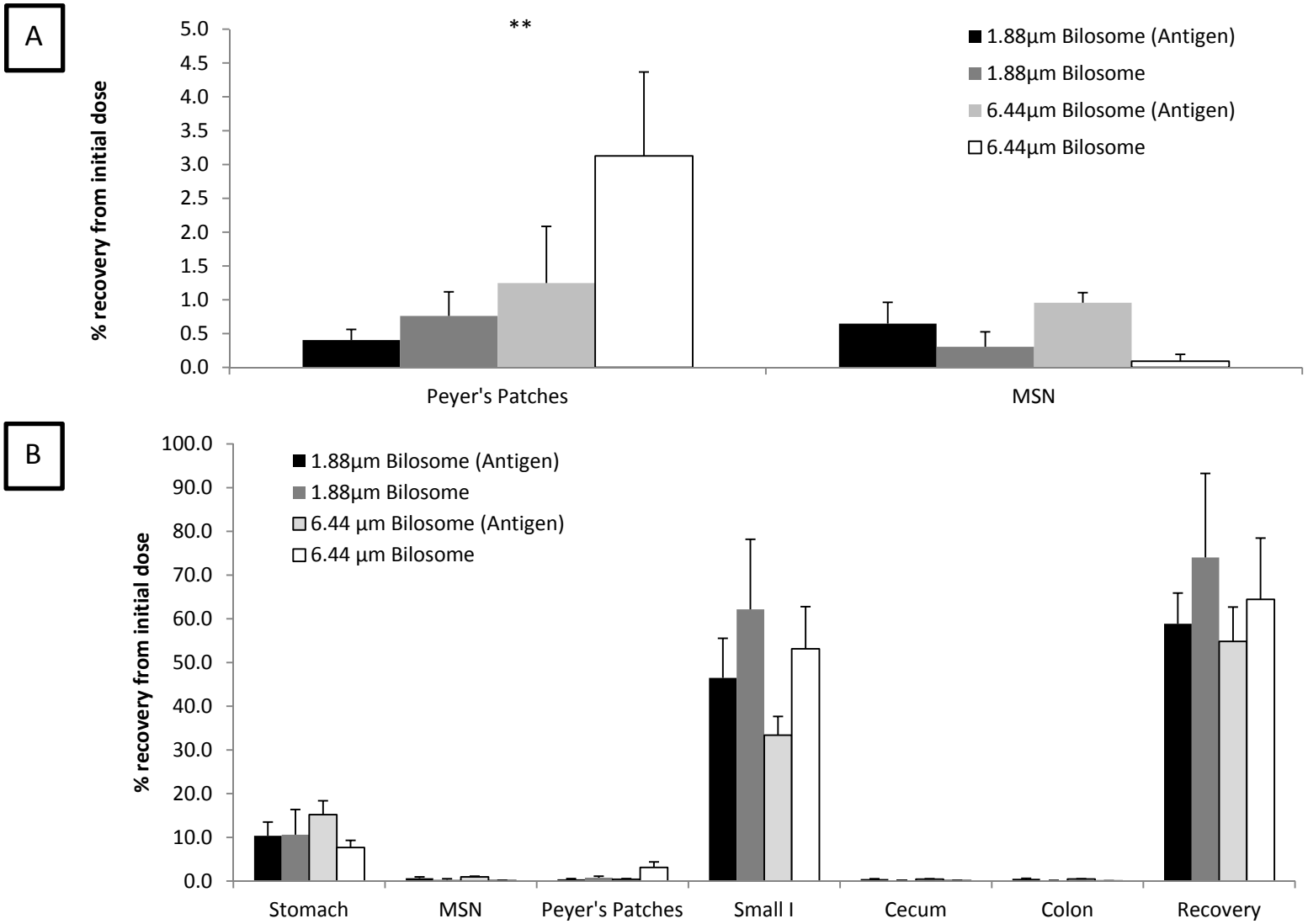

Figure 6. (A) Antigen and vesicle recovery of a reduced bilosome vesicle formulation (1.88 µm) and (B) comparative data for large and small bilosome vesicles showing antigen and vesicle recovery after a 30 min timepoint (**highly significant difference $p < 0.01$).

Oral vaccination studies

To investigate the efficacy of the optimized bilosome formulation, bilosomes containing the rHA directed against influenza viruses were administered to ferrets orally on days 0, 3, 14, and 17, and 14 days later challenged with a clinical rHA isolate of influenza. To investigate the efficacy of the vaccine and comply with 3R protocols, the key correlate of protection, the Median temperature differential, was measured to follow protection against fever and inflammatory cell counts in nasal washes (Fig 7). The bilosome vaccine incorporating the rHA shows a reduced median temperature differential change compared to a dose of empty bilosomes suggesting that the antigen containing bilosomes via the oral route provided strong protection from fever. After analysing nasal washes for inflammatory cells, the bilosome plus rHA vaccine also promoted a reduction in viral cell load counts compared to bilosomes administered without antigen. Thus the bilosome formulation containing the influenza vaccine promoted protection against fever and suppressed lung inflammation to extents comparable to empty vesicles showing promising results for vaccine delivery via the oral route.

Previous research has shown that delivering subunit antigens alone via the oral route does not sufficiently promote effective immune responses (Azizi et al., 2010). Indeed when considering recombinant influenza antigen, Amorij, *et al* (2007) have shown that the intragastric delivery of recombinant H3N2 antigen alone induced low HI titres, which were significantly lower than the antigen delivered IM (Amorij et al., 2007). This can be attributed to rapid degradation of the antigen in the GIT and/or their poor uptake by appropriate immune cells (Devriendt et al., 2012). This has been confirmed within our studies (Fig 5); the oral biodistribution of H3N2 antigen alone, shows short residence time within the GIT and low uptake in the target site. In contrast, bilosome formulated antigen promoted increased within the Peyer's patches (Fig 5 & 6) and promote effective immune responses (Fig 7).

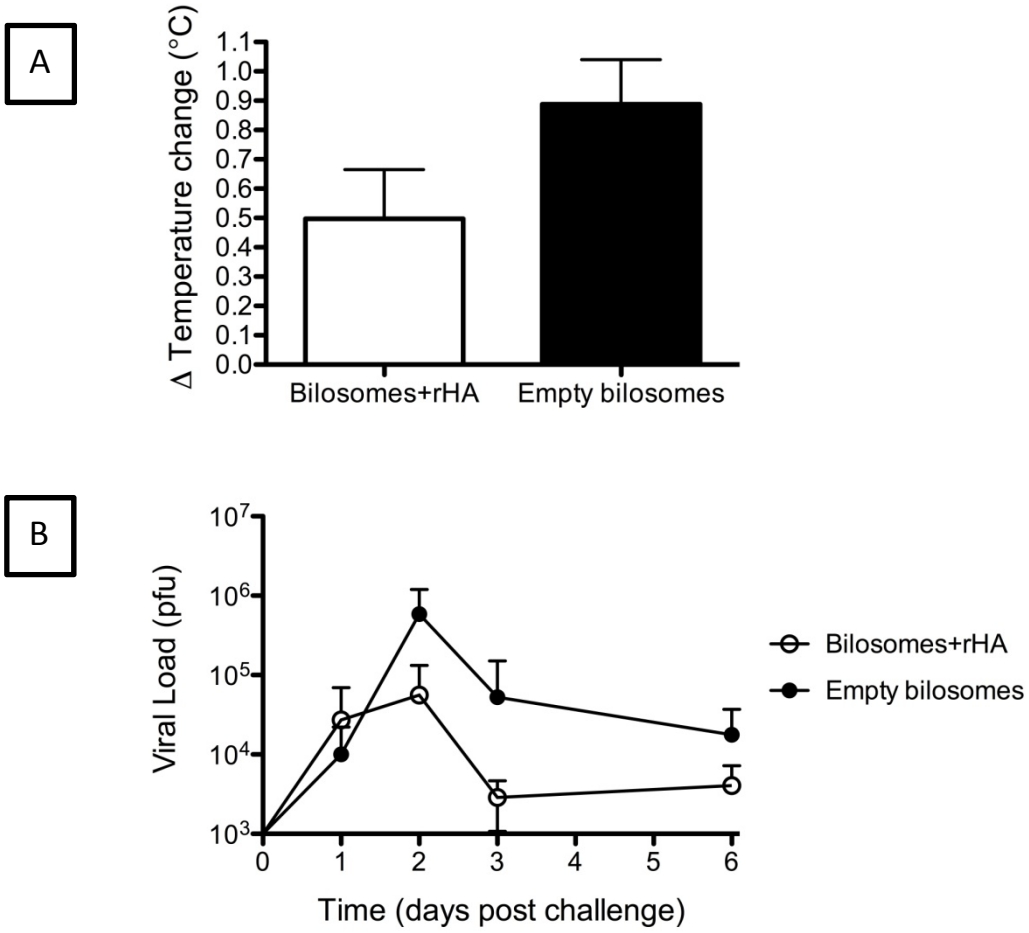

Figure 7. Orally administered bilosome vaccine containing the influenza vaccine compared to the Empty bilosome vesicles for (A) Median temperature differential (°C) and (B) total inflammatory cell count from nasal washes.

Conclusions

Whilst it is clear that oral vaccination offers a wider range of advantages, for this route to be more effectively exploited and commercialised, the key question to be addressed is how much antigen needs to be given to generate an effective immune response – when given orally, considerably higher doses of unformulated antigen is required compared to the intramuscular route. Therefore the ability of the above outlined bilosome formulation to enhance delivery of antigens directly addresses this critical point in the development of oral vaccines. Using Design of experiments an optimised bilosome formulation has been identified, which contained 5:4:1 MPG:Chol:DCP respectively with 70-120 mM Bile salt. In

terms of physico-chemical characteristics, DCP content is the parameter controlling both the zeta potential and pH suspension, as might be expected, but the addition of DCP does not alter the vesicle size; bile salt content is the controlling factor for vesicle size. The optimised bilosome formulation showed excellent antigen retention in simulated gastric conditions, with no notable loss of antigen, however when exposed to simulated intestinal conditions, 28 % of the originally incorporated antigen was lost. Using the bilosome carrier, antigen delivery to the MSN was significantly improved compared to free antigen, and vaccination of ferrets with rHA incorporated into bilosomes was able to reduce fever and suppress lung inflammation in a challenge model. Thus, the bilosome platform has shown promising results in obtaining higher immune responses obtained *in-vivo* and preventing fever in the ferret model. Further studies to optimise the bilosomes in terms of providing an antigen dose which is commercially viable are being carried out.

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References

AMORIJ, J. P., WESTRA, T. A., HINRICHS, W. L. J., HUCKRIEDE, A. & FRIJLINK, H. W. 2007. Towards an oral influenza vaccine: Comparison between intragastric and intracolonic delivery of influenza subunit vaccine in a murine model. *Vaccine,* 26**,** 67-76.

AZIZI, A., KUMAR, A., DIAZ-MITOMA, F. & MESTECKY, J. 2010. Enhancing Oral Vaccine Potency by Targeting Intestinal M Cells. *PLoS Pathog,* 6**,** e1001147.

BRAMWELL, V. W. & PERRIE, Y. 2005. The rational design of vaccines. *Drug Discovery Today,* 10**,** 1527-1534.

BRAMWELL, V. W. & PERRIE, Y. 2006. Particulate delivery systems for vaccines: what can we expect? *The Journal of pharmacy and pharmacology,* 58**,** 717-28.

CONACHER, M., ALEXANDER, J. & BREWER, J. M. 2001. Oral immunisation with peptide and protein antigens by formulation in lipid vesicles incorporating bile salts (bilosomes). *Vaccine,* 19**,** 2965-2974.

DEVRIENDT, B., DE GEEST, B. G., GODDEERIS, B. M. & COX, E. 2012. Crossing the barrier: Targeting epithelial receptors for enhanced oral vaccine delivery. *Journal of Controlled Release,* 160**,** 431-439.

EBEL, J. P. 1990. A Method for Quantifying Particle Absorption from the Small Intestine of the Mouse. *Pharmaceutical Research,* 7**,** 848-851.

ELDRIDGE, J. H., HAMMOND, C. J., MEULBROEK, J. A., STAAS, J. K., GILLEY, R. M. & TICE, T. R. 1990. Controlled vaccine release in the gut-associated lymphoid tissues. I. Orally administered biodegradable microspheres target the peyer's patches. *Journal of Controlled Release,* 11**,** 205-214.

HENRIKSEN-LACEY, M., BRAMWELL, V. & PERRIE, Y. 2010. Radiolabelling of Antigen and Liposomes for Vaccine Biodistribution Studies. *Pharmaceutics,* 2**,** 91-104.

KOGURE, K., NAKAMURA, C., OKUDA, O., HAYASHI, K. & UENO, M. 1997. Effect of dicetylphosphate or stearic acid on spontaneous transfer of protein from influenza virus-infected cells to dimyristoylphosphatidylcholine liposomes. *Biochim Biophys Acta,* 1329**,** 174-82.

MANN, J. F. S., FERRO, V. A., MULLEN, A. B., TETLEY, L., MULLEN, M., CARTER, K. C., ALEXANDER, J. & STIMSON, W. H. 2004. Optimization of a lipid based oral delivery system containing A/Panama influenza haemagglutinin. *Vaccine,* 22**,** 2425-2429.

MANN, J. F. S., SCALES, H. E., SHAKIR, E., ALEXANDER, J., CARTER, K. C., MULLEN, A. B. & FERRO, V. A. 2006. Oral delivery of tetanus toxoid using vesicles containing bile salts (bilosomes) induces significant systemic and mucosal immunity. *Methods,* 38**,** 90-95.

MANN, J. F. S., SHAKIR, E., CARTER, K. C., MULLEN, A. B., ALEXANDER, J. & FERRO, V. A. 2009. Lipid vesicle size of an oral influenza vaccine delivery vehicle influences the Th1/Th2 bias in the immune response and protection against infection. *Vaccine,* 27**,** 3643-3649.

MANOSROI, A., WONGTRAKUL, P., MANOSROI, J., SAKAI, H., SUGAWARA, F., YUASA, M. & ABE, M. 2003. Characterization of vesicles prepared with various non-ionic surfactants mixed with cholesterol. *Colloids and Surfaces B: Biointerfaces,* 30**,** 129-138.

NORRIS, D. A., PURI, N. & SINKO, P. J. 1998. The effect of physical barriers and properties on the oral absorption of particulates. *Advanced Drug Delivery Reviews,* 34**,** 135-154.

SCHMUCKER, D. L., HEYWORTH, M. F., OWEN, R. L. & DANIELS, C. K. 1996. Impact of aging on gastrointestinal mucosal immunity. *Dig Dis Sci,* 41**,** 1183-93.

SCHUBERT, R., JARONI, H., SCHOELMERICH, J. & SCHMIDT, K. H. 1983. Studies on the mechanism of bile salt-induced liposomal membrane damage. *Digestion,* 28**,** 181-90.

SHUKLA, A., KATARE, O. P., SINGH, B. & VYAS, S. P. 2010. M-cell targeted delivery of recombinant hepatitis B surface antigen using cholera toxin B subunit conjugated bilosomes. *International journal of pharmaceutics,* 385**,** 47-52.

SHUKLA, A., KHATRI, K., GUPTA, P. N., GOYAL, A. K., MEHTA, A. & VYAS, S. P. 2008. Oral immunization against hepatitis B using bile salt stabilized vesicles (bilosomes). *J Pharm Pharm Sci,* 11**,** 59-66.

SHUKLA, A., SINGH, B. & KATARE, O. P. 2011. Significant systemic and mucosal immune response induced on oral delivery of diphtheria toxoid using nano-bilosomes. *Br J Pharmacol,* 164**,** 820-7.

TOMIZAWA, H., ARAMAKI, Y., FUJII, Y., HARA, T., SUZUKI, N., YACHI, K., KIKUCHI, H. & TSUCHIYA, S. 1993. Uptake of phosphatidylserine liposomes by rat Peyer's patches following intraluminal administration. *Pharm Res,* 10**,** 549-52.

VERTZONI, M., DRESSMAN, J., BUTLER, J., HEMPENSTALL, J. & REPPAS, C. 2005. Simulation of fasting gastric conditions and its importance for the in vivo dissolution of lipophilic compounds. *European Journal of Pharmaceutics and Biopharmaceutics,* 60**,** 413-417.

WILKHU, J., MCNEIL, S. E., KIRBY, D. J. & PERRIE, Y. 2011. Formulation design considerations for oral vaccines. *Therapeutic Delivery,* 2**,** 1141-1164.