

**Title: Consideration of the efficacy of non-ionic vesicles in the targeted delivery of oral vaccines.**

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## **Abstract**

The fundamentals of this research were to exploit non-ionic surfactant technology for delivery and administration of vaccine antigens across the oral route and to gain a better understanding of vaccine trafficking. Using a newly developed method for manufacture of non-ionic surfactant vesicles (niosomes and bilosomes) lower process temperatures were adopted thus reducing antigen exposure to potentially damaging conditions. Vesicles prepared by this method offered high protection to enzymatic degradation, with only ~10% antigen loss measured when vesicles incorporating antigen were exposed to enzyme digestion. Interestingly, when formulated using this new production method, the addition of bile salt to the vesicles offered no advantage in terms of stability within simulated gastro-intestinal conditions. Considering their ability to deliver antigen to their target site, results demonstrated that incorporation of antigen within vesicles enhanced delivery and targeting of the antigen to the Peyer's Patch, again with niosomes and bilosomes offering similar efficiency. Delivery to both the Peyer's patches and mesentery lymphatics was shown to be dose dependent at lower concentrations, with saturation kinetics applying at higher concentrations. This demonstrates that in the formulation of vaccine delivery systems the lipid/antigen dose ratio is not only a key factor in production cost, but equally is a key factor in the kinetics of delivery and targeting of a vaccine system.

## Introduction

Mucosal sites are primary access points for most human pathogens, therefore the induction of mucosal immunity plays an important role to prevent pathogen entry and to prevent infection [1-3]. However, despite the oral route being easily accessible and offering good patient compliance, it is not always feasible to administer vaccines via this route due to problems of degradation of the vaccines in the gastro-intestinal tract (GIT) [4]. In addition, the residence time of vaccines at the immune induction sites within the GIT (Peyer's Patches) is short due to GI transit. The target site for mucosal vaccine delivery systems are the M cells located within Peyer's patches which are randomly distributed across the mucosa of the GIT, mainly in the jejunum [5]. Due to the short exposure time of the vaccine to the Peyer's patches, higher doses or an increase in dosing frequency are often required to supply sufficient antigen delivery to elicit an immune response [6]. However, this strategy of increasing dosing could potentially lead to reduced secretion of antigen specific IgA levels, due to increased systemic tolerance to the vaccine [7].

Currently non-ionic surfactant vesicle carrier systems, such as niosomes or bilosomes, are being employed to encapsulate/associate vaccine antigens and administer via the oral route. The addition of bile salts (to form bilosomes) has been proposed to enhance antigen delivery by offering increased protection and retention of the antigen within the bilosomes when subjected to intestinal media containing bile acids, thus preventing premature release of antigen prior to reaching the target sites [1]. Commonly, the methods used to form these vesicles involves antigens being heated to 60 °C or several freeze thaw cycles to prepare the vaccine delivery systems [1, 8] which can be detrimental. For example, Statens Serum India have shown that after three freeze thaw cycles vaccines such as Tetanus, Diphtheria and Pertussis lose up to 60% potency [9]. Similarly, the potency of a vaccine can be related to exposure to excess temperatures, therefore it is essential to maintain vaccine components at their ideal temperatures for maximum potency [10, 11].

Given these issues, it is essential that vaccines are not subjected to freeze thaw cycles or elevated temperatures as this could diminish the potency of the vaccine. Therefore we have developed a new protocol for the preparation non-ionic surfactant vesicles (with and without the addition of bile salts) at a lower process temperature incorporating thermo sensitive antigens.

This work follows on from our previously published work [12] where we studied the influence of various bilosome formulation parameters on the systemic efficacies of oral vaccine delivery. In these previously reported studies we demonstrated that the DCP content is a key formulation parameter for controlling both the zeta potential and pH of suspension and the bile salt content is the major bilosome size dictating parameter. Furthermore it was demonstrated that the larger bilosome system of 6  $\mu\text{m}$  diameter lead to enhanced uptake within the target Peyer's patches. Importantly, in an influenza challenge experiment it was shown that the orally administered bilosome system containing influenza antigen (rHA) is capable of reducing median temperature differential change and leads to significant reduction in viral cell load counts. In this paper we demonstrate a new method for bilosome production which offers an increase in antigen protection/retention within vesicles. Furthermore we demonstrate that using this method, the addition of bile salts to the formulation offers no notable advantage in terms of antigen retention or delivery after oral administration.

## **Materials and Methods**

To form the vesicles the surfactants 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 pH 7.6, where hydrochloric acid and sodium hydroxide (NaOH) (Sigma-Aldrich, UK) was used for pH adjustments. For the antigen a recombinant H3N2 sub-unit protein (Immune Tech, USA) was used.

### **Preparation of niosomes and bilosomes**

Vesicles were prepared based on a modified method [1]. Briefly, a paraffin oil bath was set up at 120 °C and a water bath at 30 °C. A 25 mM sodium bicarbonate buffer (pH 7.6) was prepared containing the H3N2 antigen and in the case of bilosomes 100 mM sodium deoxycholate (bile salt) was also added. Appropriate molar ratios of the lipids MPG, Chol and DCP (5:4:1 respectively) were weighed and placed in a 25 mL flat bottom glass beaker and the mixture was melted by heating at 120 °C for 10 minutes with occasional mixing. The molten lipids (308.5 mg) at 120 °C was immediately removed from the oil bath and an emulsion was created by the addition of the pre-incubated antigen buffered stock solution (preheated to 30 °C) and immediately homogenised (using an emulsion head) for 10 minutes at 8000 rpm. Once homogenisation had finished, the bilosome formulation was allowed to cool to 30 °C, and left for 2 hours in an incubator/shaker at 220 rpm.

### **Characterisation of vesicles.**

The size of the vesicles was determined using laser diffraction on a sympatec 2005 (Helos/BF) analyser. 20 µL aliquots of the vesicle suspension was diluted into the cuvette with 40 mL double distilled water. The zeta potential 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 vesicle suspension and left for a few minutes.

### **Removal of non-incorporated antigen**

For quantification of antigen association, ultra-centrifugation of the formulations was required to isolate vesicles with entrapped antigen, from non-incorporated antigen. To achieve this, 300 µL aliquots of sample was diluted in a Beckman 3.9 mL Polo-allomer tube and centrifuged twice at 354,000 X g for 45 minutes at 4 °C with the acceleration set at 8 (Slowest) and the deceleration at coast.

### **Radiolabelling of H3N2 antigen**

To quantify antigen incorporation, antigen release and trypsin digestion, the antigen was radiolabelled using  $^{125}\text{I}$ . To achieve this antigen stock solutions were prepared as 100  $\mu\text{g}$  (1mg/mL) in PBS stock and 40  $\mu\text{L}$  (40  $\mu\text{g}$ ) placed into an iodination tube (Pierce Biotechnology) with 2 Mbq for 1 hour and subsequently separated from non-labelled antigen by column chromatography using sephadex G75 beads [12].

### **Trypsin Digestion**

Vesicles were prepared with radiolabelled antigen and an initial antigen association was calculated by centrifugation. Trypsinisation studies were carried out by incubating aliquots of the sample with 100  $\mu\text{g}/\text{mL}$  trypsin for a period of 30 and 60 minutes at 37 °C to remove any adsorbed antigen. The samples were then centrifuged to determine surface adsorption of the antigen [14].

### **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 [15].

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 [16]. To establish the effect of these conditions on vesicle attributes, 400  $\mu\text{L}$  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 (Beckman, Ultima XP) at 354, 000 X g.

### ***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 untrapped radiolabelled antigen, were administered orally to Balb/c mice (n=4). Animals were terminated at various time points, organs collected and analysed for both <sup>125</sup>I (to quantify antigen) and <sup>3</sup>H (to quantify vesicles), which were used as a radio-active tracker for vesicles by incorporating <sup>3</sup>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 <sup>125</sup>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 the 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.

### **Confocal laser scanning microscopy**

Niosome vesicles were prepared incorporating a Dil-C lipid dye which was co-melted during the lipid melting phase of the method. The antigen was pre-labelled with a fluorescent flamma fluor FPR-648 (Bio Acts) which was carried out by incubating the fluorescent marker with the antigen and then

separated using a P-10 (10Kd MWCO) centrifugation tube to remove unbound marker. The niosome vesicles were prepared and administered orally to Balb-C mice and after 30 minutes the Peyer's Patch was spliced along with the mesenteric lymph tissue to locate the vesicles and the antigen. Samples were then analysed on a Leica Confocal microscope using a 63x objective.

### **Statistical analysis**

The results within this study are given as the geometric mean  $\pm$  S.D. unless stated otherwise. The statistics were carried out using ANOVA and a probability factor of less than 0.05 ( $P < 0.05$ ) was considered to represent statistically significant difference.

## **Results and Discussion**

### **Physical characteristics of vesicles along the Gastro Intestinal Tract**

Non-ionic surfactant based vesicles have provided effective immunity in various animal models such as mice and ferrets after oral administration [12, 17]. The oral route provides a challenging environment for delivery of antigens including low pH, digestive/ gastric enzymes the poor absorption and the rapid transit [18]. As a result, it is important to ensure that the antigen and carrier system remains intact during transit through the GI tract until it reaches the target site. Therefore to investigate this, vesicles were subjected to simulated gastric media (pH 1.2) and either transfected from gastric media to simulated intestinal media (pH 8.6) or placed directly into simulated intestinal media and at various time points the vesicles were analysed for their characteristics (vesicle size, zeta potential and antigen retention; Figure 1).

Results (Figure 1A) show that the niosome preparation significantly decreases ( $p < 0.05$ ) in volume mean diameter (VMD) from  $6.54 \pm 0.04 \mu\text{m}$  ( $t = 0$  h; prior to exposure to gastric media) to  $5.46 \pm 0.05 \mu\text{m}$  after 1 h gastric media, down to  $3.57 \pm 0.03 \mu\text{m}$  after 4 hours in GSIF medium. In contrast, bilosome vesicles significantly increase in VMD ( $p < 0.05$ ) from  $6.19 \pm 0.04 \mu\text{m}$  to  $9.13 \pm 0.31 \mu\text{m}$



when in gastric medium for 1 h yet they return to their original vesicle size when placed back in SIF (to  $6.11 \pm 1.27 \mu\text{m}$ ;  $t = 4 \text{ h}$ ; Figure 1A). Directly adding the vesicles into SIF made no significant difference to the size of the niosomes or the bilosomes (Figure 1A).

Considering the vesicle zeta potential, results (Figure 1B) show that whilst the both niosome and bilosome vesicles are highly negative in nature at neutral pH ( $t = 0 \text{ h}$ ) as would be expected given their DCP content, in media at pH 1.2 a notable reduction in zeta potential occurs (from  $\sim -100 \text{ mV}$  to  $-35 \text{ mV}$ ; Figure 1B) and when in conditions as would be at the site of uptake (intestinal regions containing Peyer's Patches; pH 8.6), the zeta returns to its original value of  $-100 \text{ mV}$  (Figure 1B). This trend is observed for both niosomal and bilosome formulations.

These changes in size may be due to differences in osmotic pressure and/or flocculation properties of vesicles within the various media, particularly given the changes in zeta potential resulting from the differences in electrolytes present. Similar to these findings, an increase in carrier size has also been observed by Mane and Muro (2012) where nanocarriers containing IgG incubated in acidic simulated gastric fluid showed an increase in carrier size which was not observed in neutral simulated intestinal fluids [19]. The authors attributed the increase in size to be due to the degradation of IgG resulting from pH changes which caused aggregation. Given that studies have shown the proposed transparacellular uptake of particles within the Peyer's patches is limited to below  $10 \mu\text{m}$  [20-22] it is important that the vesicle size and surface characteristics remain appropriate for this route. In addition, it has been suggested that the retention of particles within the Peyer's patches is optimum for vesicles  $4 -10 \mu\text{m}$  in size, whilst vesicles below  $3 \mu\text{m}$  translocate into the lymphoid tissue [21, 23, 17]. This is also supported by a study using microspheres by Tabata et al, (1996) which has shown that particles within the  $5-10 \mu\text{m}$  induced a mucosal immune response whereas smaller vesicles migrate into the lymphatics resulting in systemic immunity [24]. The results in Fig 1A and B show that whilst the vesicle size and zeta potential is dependent on the media the

vesicles were suspended in, when suspended in conditions simulated to represent the environment of the target area for vaccine uptake the vesicles fall within an acceptable range for uptake and retention within the Peyer's patches.

Whilst the physico-chemical characteristics were appropriate for particulate uptake by the Peyer's patch, it is also pivotal that the vesicles are able to carry antigen to this target site. To consider this, the H3N2 antigen was radiolabelled and incorporated into the niosome and bilosomes vesicles and antigen retention studies were carried out. Considering initial antigen (Figure 1C), there was no notable difference in antigen incorporation between niosomes and bilosomes ( $39.21 \pm 2.72$  vs  $32.5 \pm 2.9$  % for niosomes and bilosomes respectively; Figure 1C). Considering the impact of the GI environment, results in figure 1C indicate that after 15 min in gastric medium (pH 1.2) niosome antigen retention decreased by approximately 10 % (from  $39.21 \pm 2.72$  to  $28.04 \pm 0.95$  %; Figure 1C) and then remained around this level for up to 60 min in gastric media. In contrast, bilosomes show no significant loss in antigen retention when incubated in gastric media for up to 60 min (maintaining their antigen retention levels of  $\sim 32$  %; Figure 1C). When the vesicles are taken from gastric to intestinal medium, both the niosome and bilosome formulations showed reductions in antigen retention (Figure 1C), with only  $\sim 10$  to  $15$  % of the antigen being retained. This reduction in antigen loading was a result of the vesicles being suspended in the higher pH intestinal media, with prior exposure to gastric media making no notable difference as demonstrated by the fact there was no difference in antigen loading for vesicles first suspended in gastric media and then transferred to SIF, compared with vesicles suspended directly into SIF (Figure 1C).

To consider the special location of the antigen and their ability to protect antigen from enzymatic degradation, both niosomes and bilosomes were subjected to protein (trypsin) digestion. Figure 2 shows that after incubation with trypsin, both formulations show low antigen loss ( $\sim 5$  %) suggesting that in both systems the antigen is predominately located within the vesicles (and hence protected from protease digestion). Considering the results of Figure 1C and 2, the choice of bilosome over

niosome gives no advantage with antigen recovery being similar in both formulations. This is in contrast to previous studies by Conacher *et al.*, (2001) where they demonstrated that bilosomes retained a higher percentage of their entrapped bovine serum albumin compared to the corresponding niosomes when subjected to 20 mM bile salt concentrations [1]. However, they confirmed that the presence of bile salts within the formulations did not increase adjuvant activity and stimulated similar immune responses to niosomes.

In this present study, the method of manufacture of the vesicles has been modified to reduce the temperatures used in the manufacturing process and also to potentially promote higher loading within the aqueous core of the vesicles by adding the antigen at an earlier stage during the homogenisation procedure. Therefore, by modification of the manufacture method (Figure 3), the results in figure 1 and 2 suggest that the addition of bile salts does not improve antigen protection. Indeed in previous studies from our laboratory [17] using bilosomes formulated by the method of Conacher *et al.*, (2001) whilst in general vesicle size and zeta potential were similar for the two types of manufacture, we have measured a sharp decline in antigen recovery when placed into the SIF after gastric exposure; in these previous studies [17] only ~ 7-8 % antigen was associated with bilosomes (of comparable surfactant composition), compared to significantly higher ( $p < 0.05$ ) antigen association of ~ 10-15 % (Figure 1C) when bilosomes are formulated using the currently reported bilosome production method (which adopts lower temperatures and includes antigen at the start within the aqueous buffer; Figure 3).

#### **Visualisation of vesicles in GI transit via confocal laser scanning microscopy**

Within this study we have shown that the vesicles remain within the desired size range 5-10  $\mu\text{m}$  after subjecting to simulated gastric and intestinal conditions. To obtain a greater understanding of antigen/vesicle transport through the GIT, the bilayers of the vesicles were fluorescently labelled with a lipid dye (Dil-C) and the antigen was fluorescently labelled with a flourophore dye. Figure 4

represents the imaging process from manufacturing of the vesicles to the site of uptake of the vesicles/ antigen *in-vivo*.

As represented within figure 4, the vesicles have a strong red colour (Figure 4A) and the antigen (labelled blue) resides within the aqueous core of the vesicles, which can be compared to vesicles without antigen that show a black aqueous core. These fluorescently labelled vesicles were administered via oral gavage (0.2 mL dose; [25, 26]) and subsequently after 30 minutes tissues were excised and analysed via microscopy to locate the vesicles. Figure 4C shows the removed GIT 30 min after oral administration of the vesicles, and upon excising of the Peyer's patch intact vesicles that are spherical and between 5-10  $\mu\text{m}$  in size can be observed in large numbers when on a low magnification (Figure 4D). Upon increased magnification, and 40  $\mu\text{m}$  deep within the tissue, a 5  $\mu\text{m}$  vesicle is clearly visible (Figure 4E), suggesting that the Peyer's patches uptake the vesicles. Moving from the Peyer's patches to the underlying mesenteric lymph tissue (Figure 4F) once again fluorescence is visible with a green autofluorescence appearing from collagen within the capillary network within the mesentery.

### ***In-Vivo* biodistribution of vesicles and their associated antigen**

Dual radiolabelling techniques have previously been used to follow the movement of vaccine antigens and their delivery systems (e.g. [12]). It is important to understand the biodistribution of mucosal vaccines administered orally as they are subject to dilution in mucosal secretions, captured within the mucus and attacked by the mucosal proteases and enzymes with the largest obstacle being exclusion by the epithelial barriers [27]. Within this study, the location and biodistribution of H3N2 antigen ( $^{125}\text{I}$ ) and the vesicle carrier system ( $^3\text{H}$ - Chol) was used to consider the uptake of vesicle and antigen and to determine the residence time of the vaccine within the GIT. Again oral gavage volume of 0.2 mL was used.

To consider if the addition of bile salt to the niosomes prepared by the revised method (as outlined in figure 3) offered advantages in terms of antigen delivery, the biodistribution of bilosome and niosome formulations were analysed at 0.5, 1 and 4 h after administration. Throughout the study the blood, spleen, kidneys and liver had only trace levels of antigen and vesicles detectable (data not shown) with the majority of the both being located in the stomach, small intestine, the colon and cecum (Figure 5).

When considering carrier (niosomes or bilosomes) distribution, the majority of the dose was located within the stomach and small intestine (Figure 5A); around 30 % of the initial dose was detected within the small intestine after 0.5 h with no significant difference between the two formulations. By 4 h the levels in the small intestine had decreased with higher levels being detected in the cecum and colon, as would be expected for the formulations as they transit through the GIT. Again there was no significant difference in the profiles of the two formulations (Figure 5A). Considering the transit of the associated antigen (Figure 5B), whilst comparable levels of 'free' and vesicle-incorporated antigen are found within the stomach, significantly lower levels (< 10 % of initial dose) for 'free' antigen was detected in the small intestine after 1 h compared to antigen carried by vesicles (Figure 5B). Moreover these higher levels of antigen recovery correlated with vesicle recovery as it transits from the stomach and small intestine, and then over time the dose progressed down the GIT to the cecum and colon (Figure 5A and B) suggesting the antigen has remained associated with the vesicles. Again there was no significant difference in the profile of the niosome and bilosome delivered antigen across the study (Figure 5B).

### ***Targeting of the vaccines to the Peyer's Patch and mesenteric lymph tissue***

The Peyer's patches are located at the antimesenteric border of the intestine where they appear as nodular white masses (1.5 – 3.0 mm) and are key to antigen uptake and induction of immunity [28]. The Peyer's patches are an attractive route for targeted delivery as this provides a direct route for antigen to reach the lymphatic system where first pass metabolism is avoided and the chances of

cellular rejection are reduced due to the M cells not expressing P-glycoprotein efflux pumps [29, 30]. Furthermore, the Peyer's patches, in comparison to the rest of the GI mucosa, possess minimal mucosal coating where transcytotic activity is high [31, 32]. To consider targeting of the vaccine system to the Peyer's patch approximately 8-11 Peyer's patches recovered (in line with literature a number of 6-12 patches; [28]). Figure 6A shows that using a vesicle carrier system made no significant difference to the uptake of antigen within the Peyer's patch and there was no significant difference in carrier uptake between the niosomes and bilosomes (which was in the range of 1-5 %; Figure 6A). However in terms of antigen recovery within the mesenteric lymph tissue, the use of a vesicular delivery system significantly ( $P < 0.01$ ) increased antigen delivery, yet the choice of carrier (niosomes versus bilosomes) made no significant difference to either antigen or carrier localisation within the mesenteric lymph tissue (Figure 6B).

The vesicles used within this study were shown (Figure 1) to remain within the desired size range for increased uptake and retention within the Peyer's patches [17]. Figure 6 indicates that the vesicles and antigen are able to penetrate and reside within the lymph tissue for a period of 4 hours. This is beneficial as the vesicles which remain in the Peyer's patches offer increased mucosal immunity whereas the increased antigen and vesicles within the mesentery is more likely to induce systemic immunity thus, offering both mucosal and systemic immunity [24]. In addition, Neutra and Kozlowski (2006) determine that antigens delivered to mucosal sites within the small intestine offer greater IgA secretions which are a characteristic of mucosal immunity compared to antigen delivery via routes such as nasally where a systemic antibody response is achieved, due to the migration of antigen to the draining lymph nodes [27].

### **The impact of carrier dose on antigen uptake**

Based on the biodistribution study a question arose; is uptake within the Peyer's patches limited in terms of saturation and uptake? To address this, a study considering varying doses of the vaccine was carried out which involved four groups of a) double dose (antigen 180  $\mu\text{g}/\text{mL}$ , lipid 27  $\text{mg}/\text{mL}$ ),

b) a standard dose from Figure 5 & 6 (antigen 90 µg/mL, lipid 13.5 mg/mL), c) a half dose (antigen 45 µg/mL, lipid 6.75 mg/mL) and d) a quarter dose (antigen 22.5 µg/mL, lipid 3.375 mg/mL) and uptake of vesicles and antigen at the target site Peyer's patches and Mesenteric lymph tissue considered. Based on the biodistribution study the organs removed for the following study focussed specifically on the GI tract which included the stomach, Mesenteric lymph tissue, Peyer's patches, small intestine, colon and cecum (Figure 7).

Results from the study (Figure 7) show no significant differences in percentage antigen or vesicle recovery between different dose concentrations in the organs collected after a 30 min time point and overall percentage recovery of antigen is comparable (40-70 %) between all doses administered based on the initial dose (Figure 7A). The vesicle recovery (Figure 7B) between the organs is also comparable, with no significant differences suggesting that the clearance rate and the gastric emptying time was not formulation dose dependent over the range tested. Gastric emptying time has been attributed to the fed or fasted state of the GIT, where the gastric emptying time ( $T_{1/2}$ ) in the fasted state is up to  $2 \pm 1$  min compared to a fed state  $T_{1/2}$  of  $17 \pm 2$  min in a mouse model [33]. Whilst both the niosomes and bilosomes were shown to have similar stability, previous studies have shown that the stability of lipidic systems can influence gastric clearance. For example, Marciani et al, (2009) study the stability of lipid emulsions on gastric emptying; they demonstrate variations in gastric emptying times with 500mL of (15% w/w) [ $^{13}\text{C}$ ] palmitate-enriched olive oil-in-water emulsion meals depending on the lipid composition [34]. Acid-unstable lipid emulsions broke down and rapidly layered in the stomach compared to acid-stable lipid emulsions which had significantly slower gastric emptying. The acid-stable emulsion emptied from the stomach linearly compared to the acid-unstable emulsion which emptied exponentially. The authors attributed this to acid-stable emulsion inducing increased fullness, decreased hunger and decreased appetite, where it was concluded that it is possible to delay gastric emptying and increase satiety by stabilising the intragastric distribution of lipid emulsions against the gastric acid environment [34].

When considering concentrations of lipid and antigen uptake rather than percentage dose (Figure 8), there is a general trend of increased concentrations of antigen and carrier within both the Peyer's Patches and the mesentery lymph tissue as the dose increases up to 90 µg/mL antigen, 13.5 mg/mL lipid (figure 8). This suggests that increasing the dose of a vaccine can improve delivery to the Peyer's patch and mesentery lymphatics. However a saturation point may be reached as shown in figure 8B, where the increase in the dose to 180 µg/mL antigen / 27 mg/mL lipid did not significantly increase delivery. Using lipid-based delivery systems has been demonstrated to increase the lymphatic transport and absorption of drugs in other studies. For example, lymphatic uptake of halofantrine was increased when it was incorporated within a self-microemulsifying drug delivery system based on structured triglycerides containing medium and long chain fatty acids [35, 36]. The lipid content within the drug delivery system was able to trigger sufficient lymphatic transport of halofantrine thus, increasing bioavailability. Saturation of uptake via this route has also been demonstrated by Florence et al, (2000) using polylysine dendrimer coated with a lipid surface; their results showed maximum uptake within Peyer's patches of 1 % after a period of 3 hours which was not increased upon increased dose concentration levels [37].

## **Conclusions**

The proposed method of producing vesicles to protect thermolabile antigens by lowering the process temperatures shows that antigen can be associated and entrapped within non-ionic surfactant vesicles. Confocal laser scanning microscopy confirms that the vesicles are spherical in nature and the vesicles meet the criteria of being within the optimum size range and charge for increased uptake within the target site of the Peyer's Patches. The biodistribution studies demonstrate that by adopting the new production method for these non-ionic surfactant vesicles, the necessity of the addition of bile salts to enhance protection and delivery is circumvented as both antigen retention and antigen delivery to the target site after oral administration was comparable.



However, uptake at the target site was shown to be dose-rate limited with saturated uptake becoming apparent and therefore antigen to lipid dose ratio may be a key factor not only in terms of cost effectiveness but also in vaccine efficacy, and thus requires further consideration in the assessment of the design of carrier-mediated immunisation.

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All institutional and national guidelines for the care and use of laboratory animals were followed. The experiments within this study comply with the current laws within the United Kingdom.

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### Figure legends:

Figure 1: Characterisation of niosomes and bilosomes when subjected to GI fluids at various time points for A) Vesicle size, B) zeta potential and C) antigen retention within the vesicles (n=3).

Figure 2: Antigen protection from enzyme digestion offered by incorporation within vesicles by trypsinisation (n=3).

Figure 3: Schematic representing method modifications and corresponding characterisation data between bilosomes produced via the pre-formed vesicle method as outlined Conacher et al., 2001 or the method outlined within the Method section where antigen is incorporated within the vesicles in-situ.

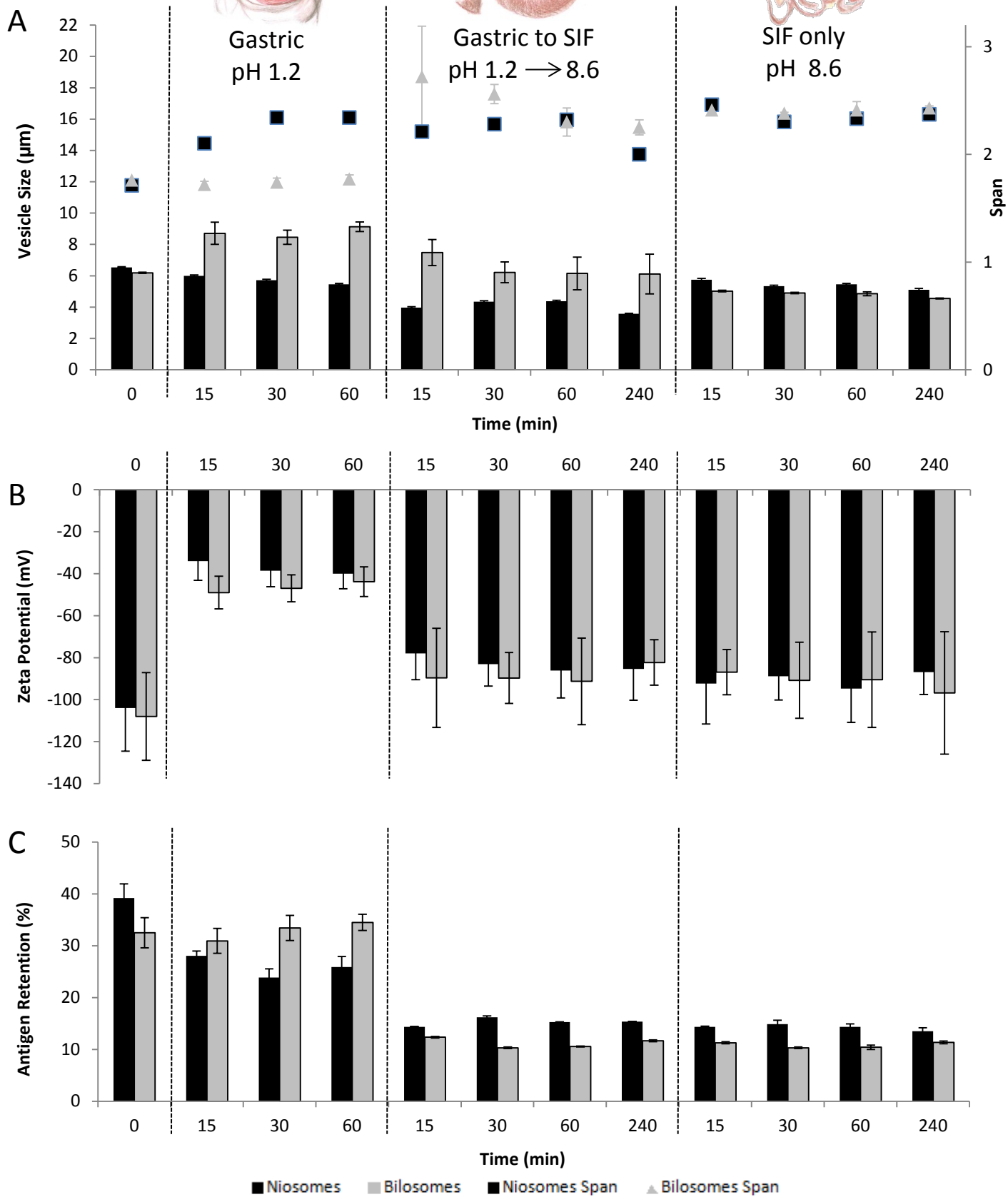
Figure 4: Confocal laser light microscopy images of niosome vesicles labelled with a lipid Dil-C dye and antigen labelled with a Flammaflour FPR 648 dye. Stages include A) vesicle emulsion upon formation, B) analysis of vesicles representing vesicles corresponding to vesicle size data showing antigen location, C) vesicles in GI transit, D) spherical vesicles within Peyer's patches, E) vesicle within Peyer's patch 40  $\mu\text{m}$  deep and F) mesenteric lymph tissue fluorescence.

Figure 5: *In vivo* trafficking of antigen and vesicles along the GIT after oral gavage where A) represents the niosomes or bilosomes radiolabelled with  $^3\text{H}$ -Cholesterol and B) represents H3N2 antigen radiolabelled with  $\text{I}^{125}$  and dosed without a carrier system or in associated with bilosomes or niosomes (n=4 at each time point).

Figure 6: Recovery of antigen and vesicles based on initial dose at  $t= 1\text{h}$  within A) Peyer's patches and B) Mesenteric Lymph Tissue (n=4).

Figure 7: *In vivo* saturation study of various dose concentrations representing antigen and vesicle recovery within the organs selected; A) antigen recovery, B) vesicle carrier recovery (n=4).

Figure 8: Representation of dose recovery per mL at site of uptake for various dose concentrations of the formulation within Peyer's patches and mesenteric lymph tissue; A) antigen dose and B) lipid dose (n=4).



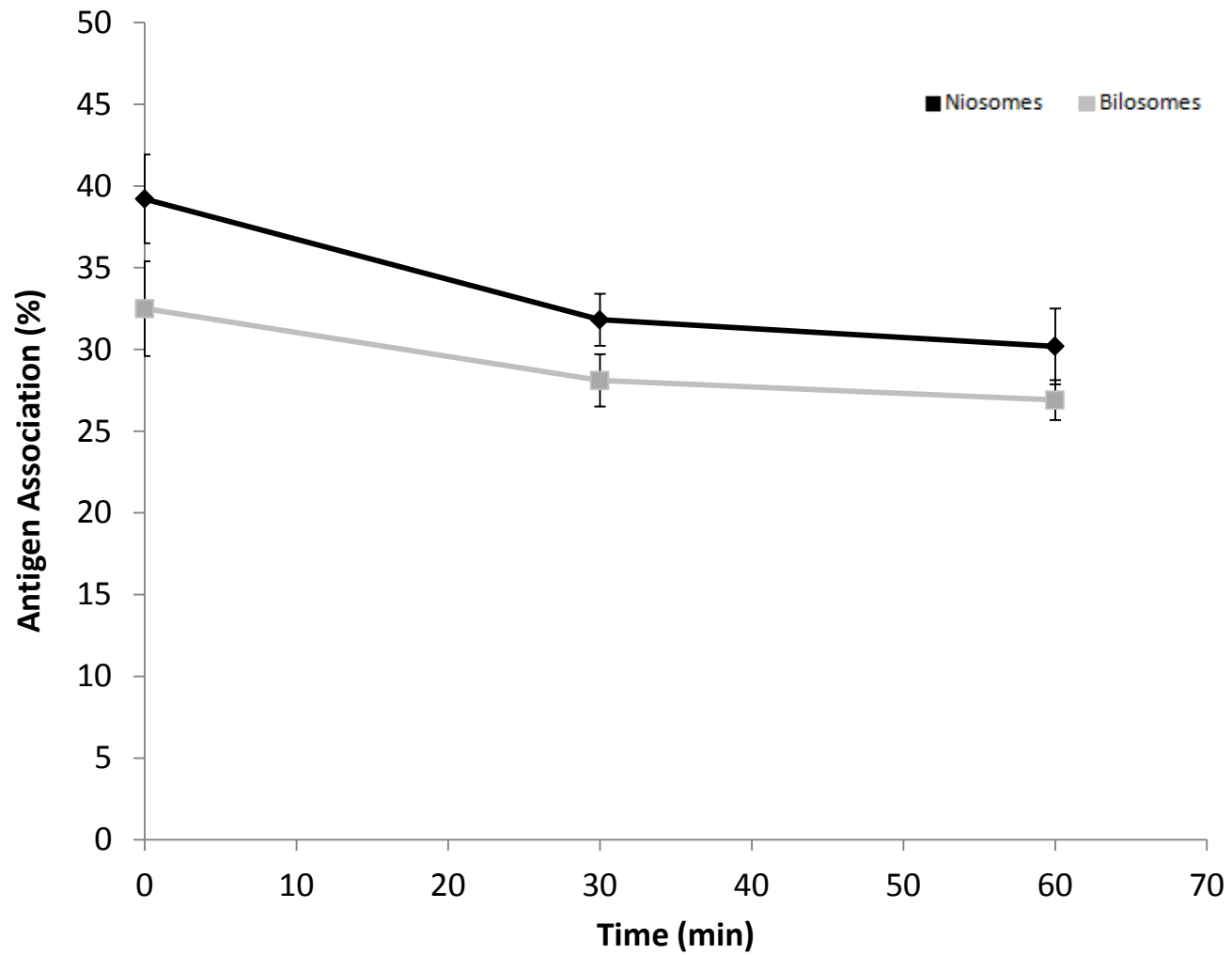
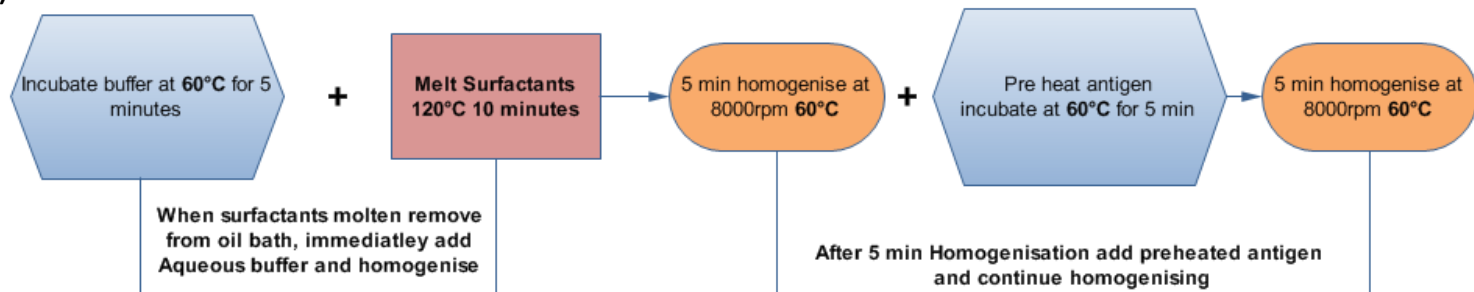
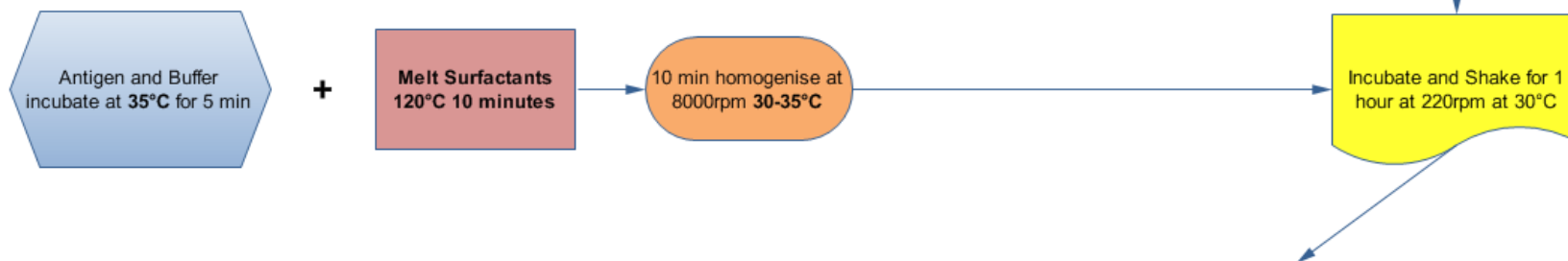


Figure 2

### A) Pre-formed vesicle method



### B) Antigen incorporation in-situ



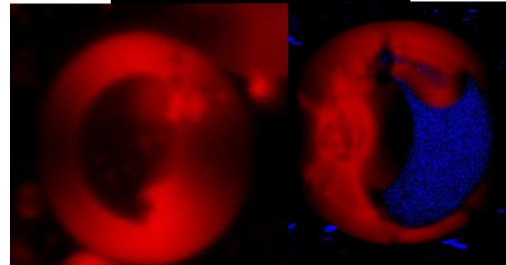
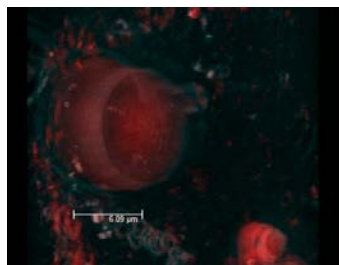
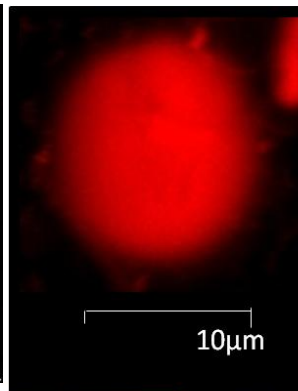
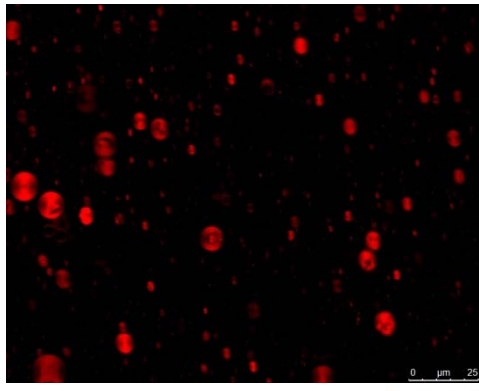
Attribute	A) Pre-formed vesicle method	B) Antigen incorporation in-situ
Vesicle Size ( $\mu\text{m}$ )	$6.44 \pm 0.5$	$6.19 \pm 0.12$
Span	1.76	1.71
Zeta Potential (mV)	$-100 \pm 12$	$-108 \pm 17$
Antigen Retention (%)	$32.3 \pm 1.2$	$32.5 \pm 2.9$
Antigen Retention after SIF	$7.1 \pm 0.1$	$12.5 \pm 0.2$

Figure 3





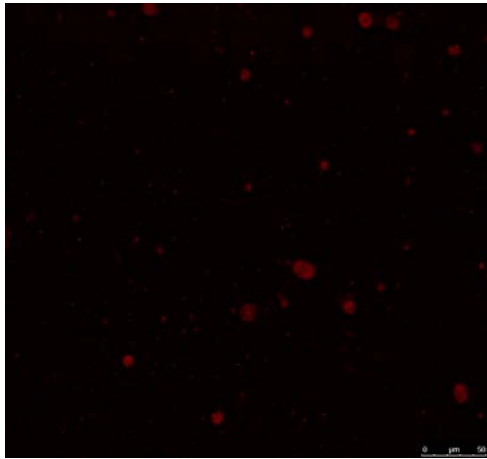
A) Vesicles



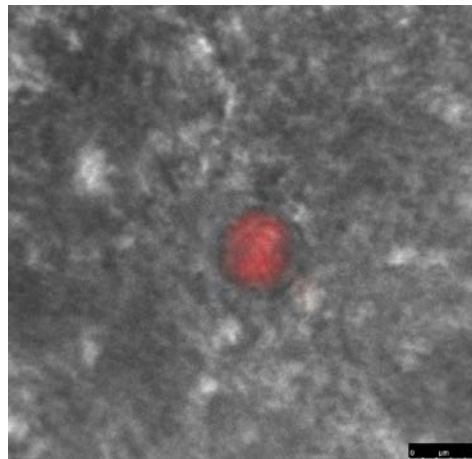
B) Confocal light microscopy showing vesicles



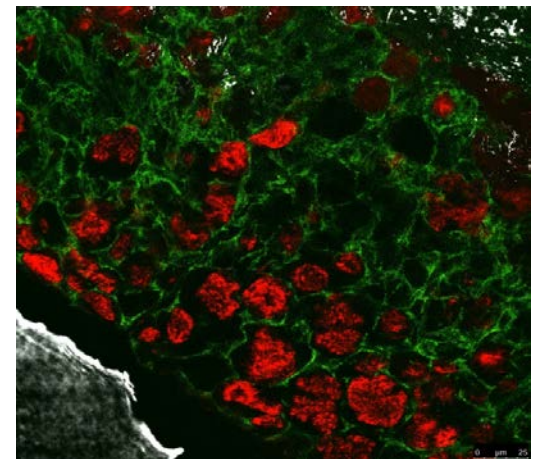
C) GI Transit



D) Peyer's Patches showing spherical vesicles present,



E) Peyer's Patch 40 micrometers deep showing spherical vesicle present



F) Mesenteric Lymph Tissue

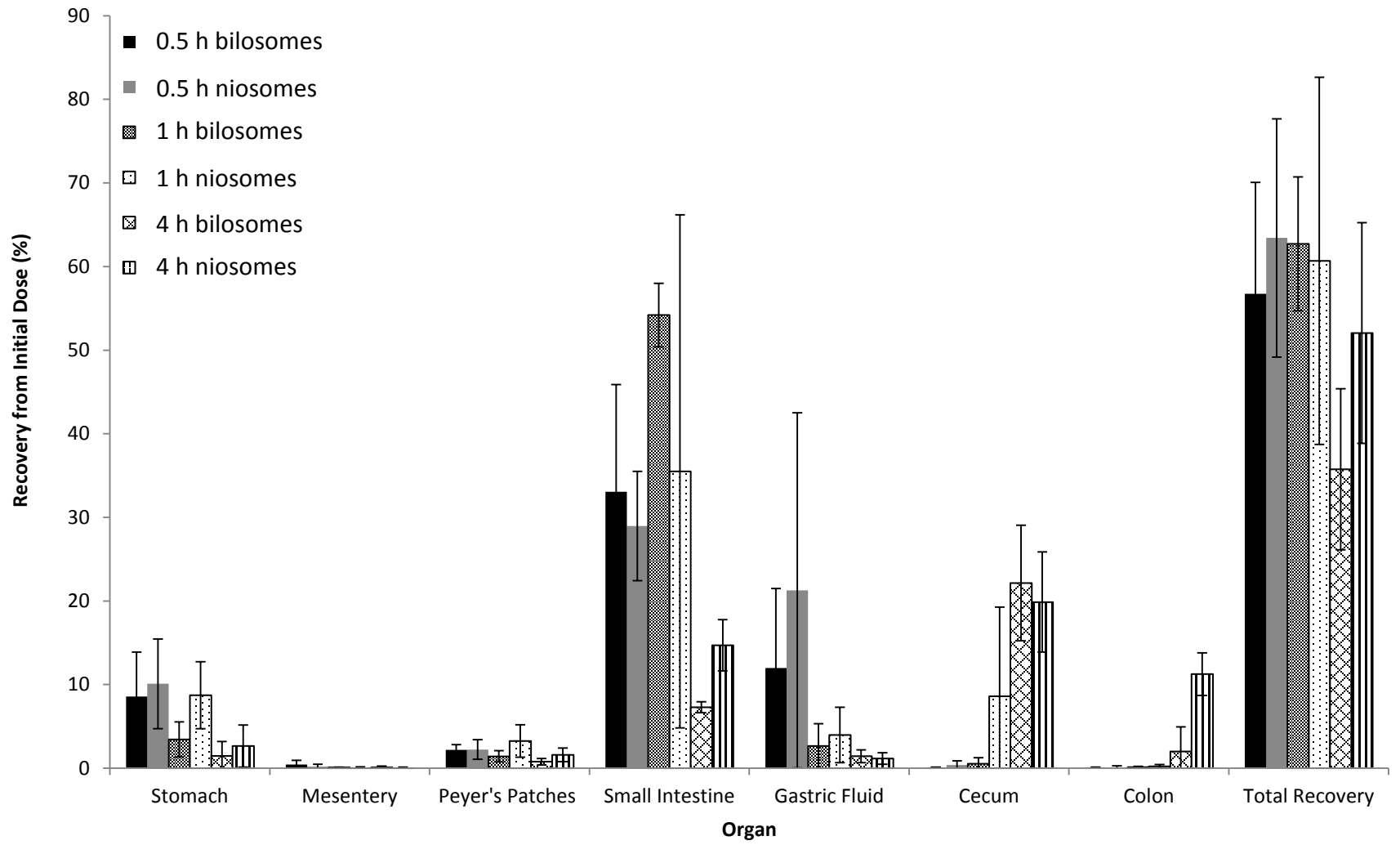
**A**

Figure 5A

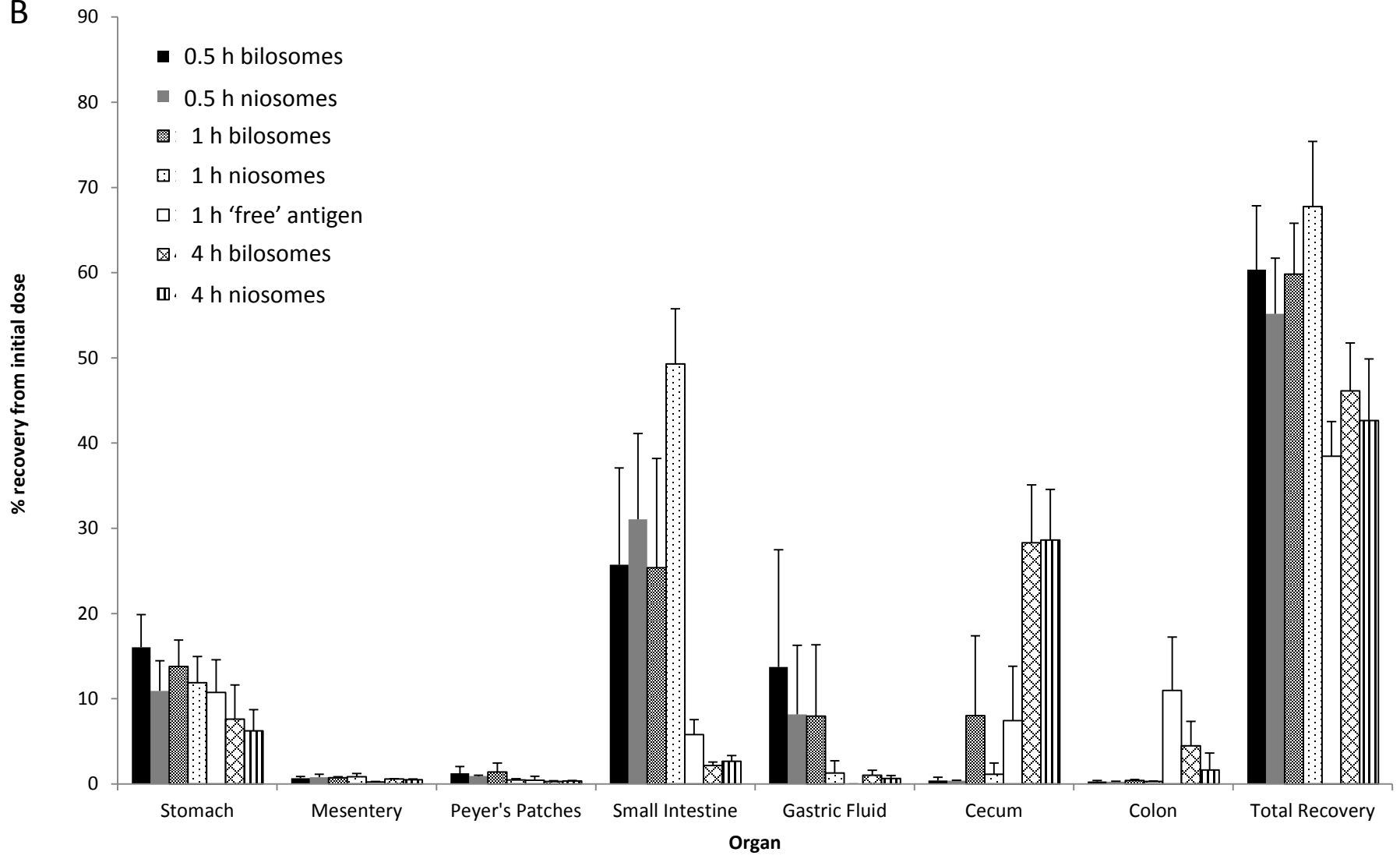
**B**

Figure 5B

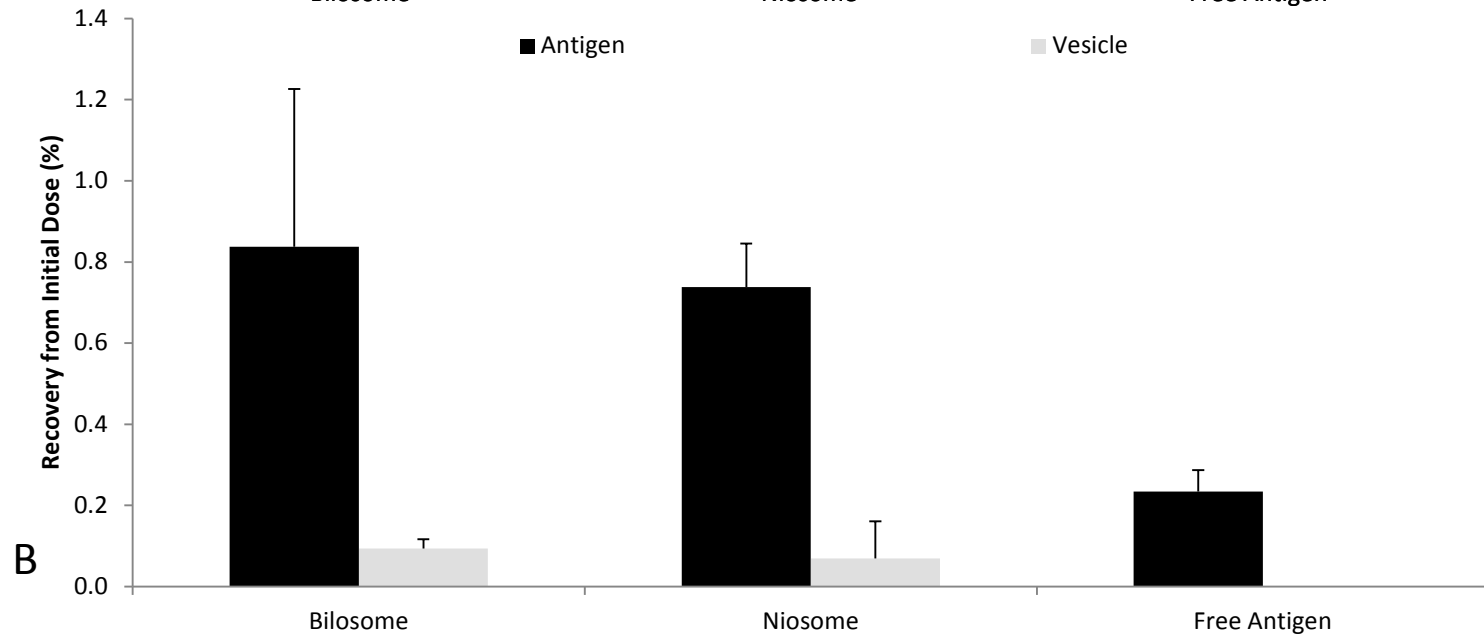
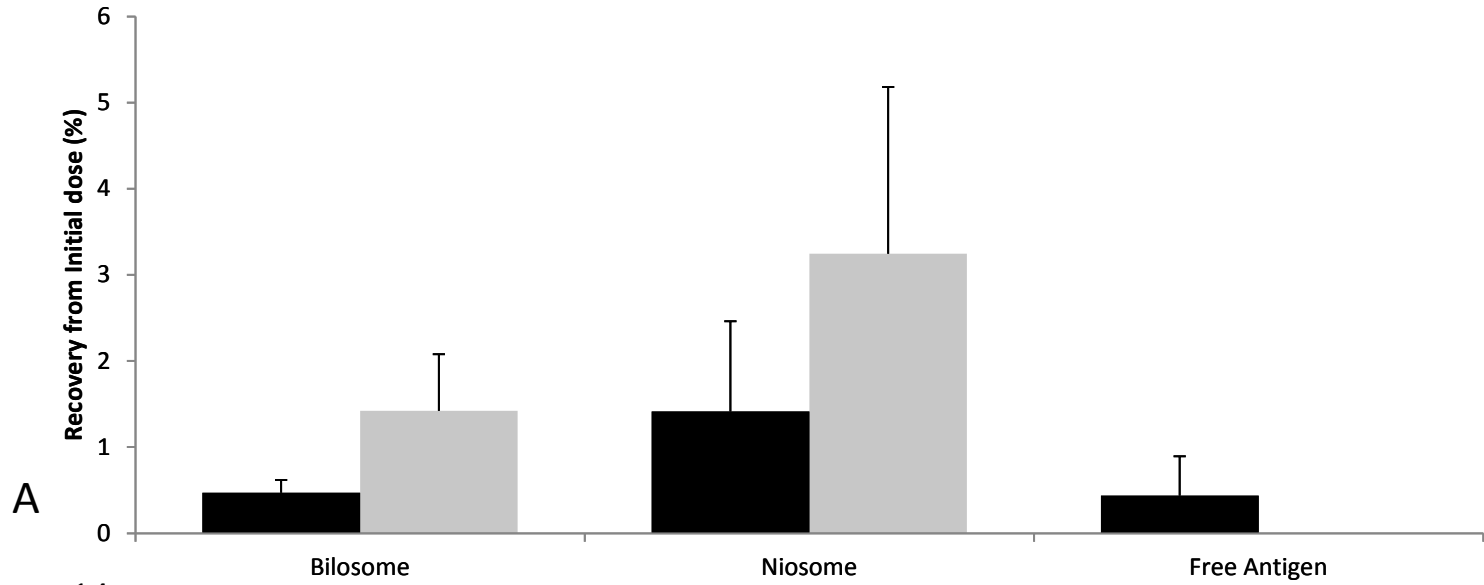


Figure 6

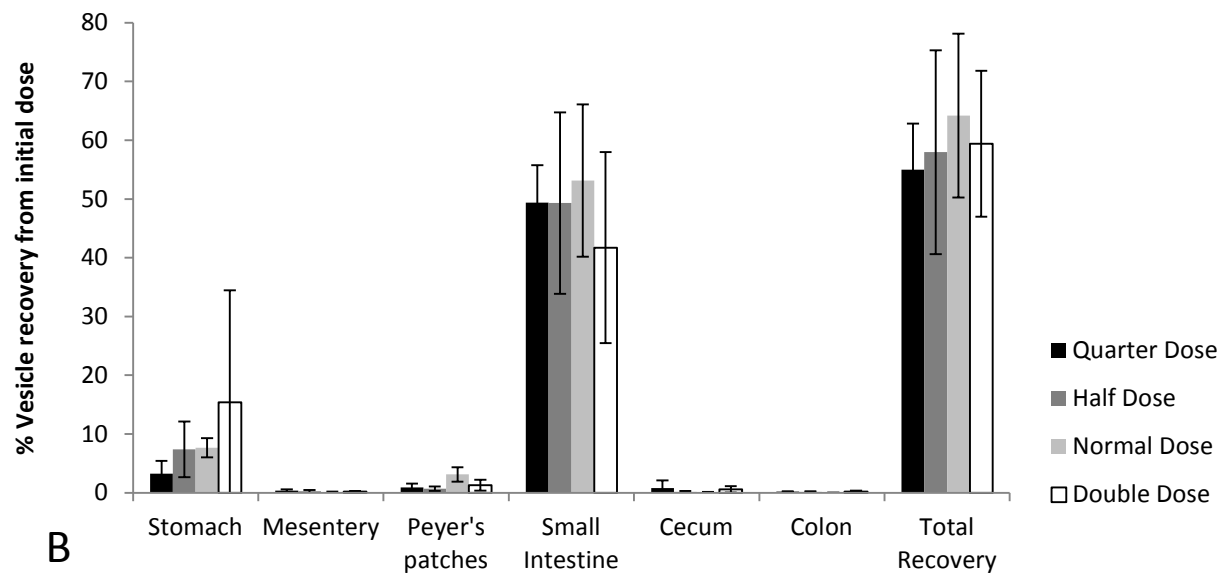
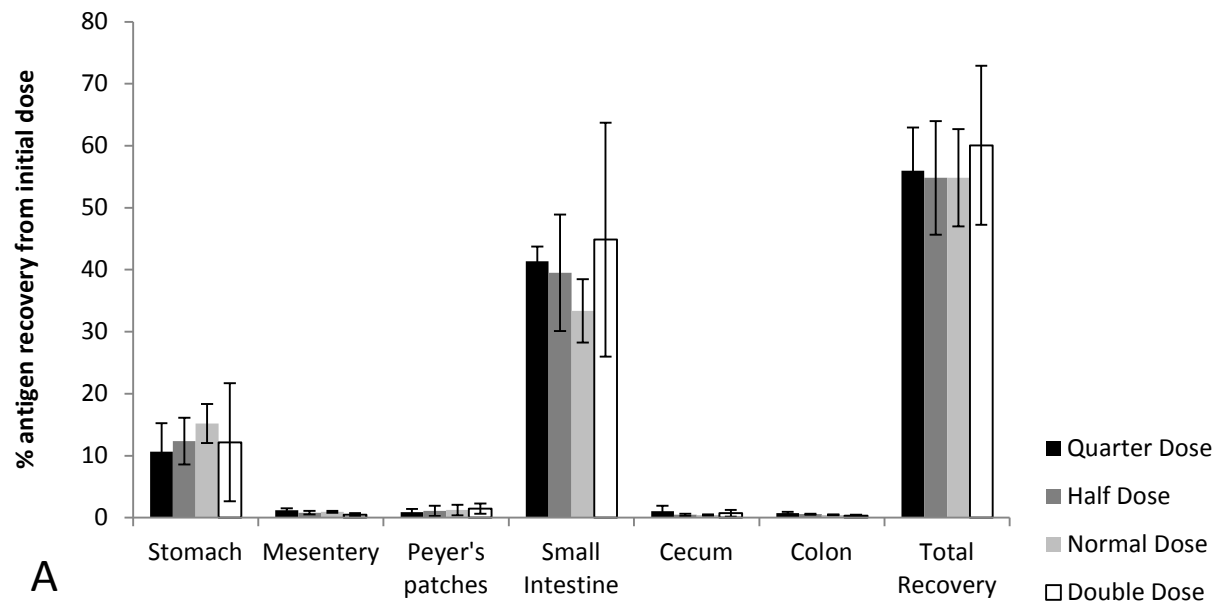


Figure 7

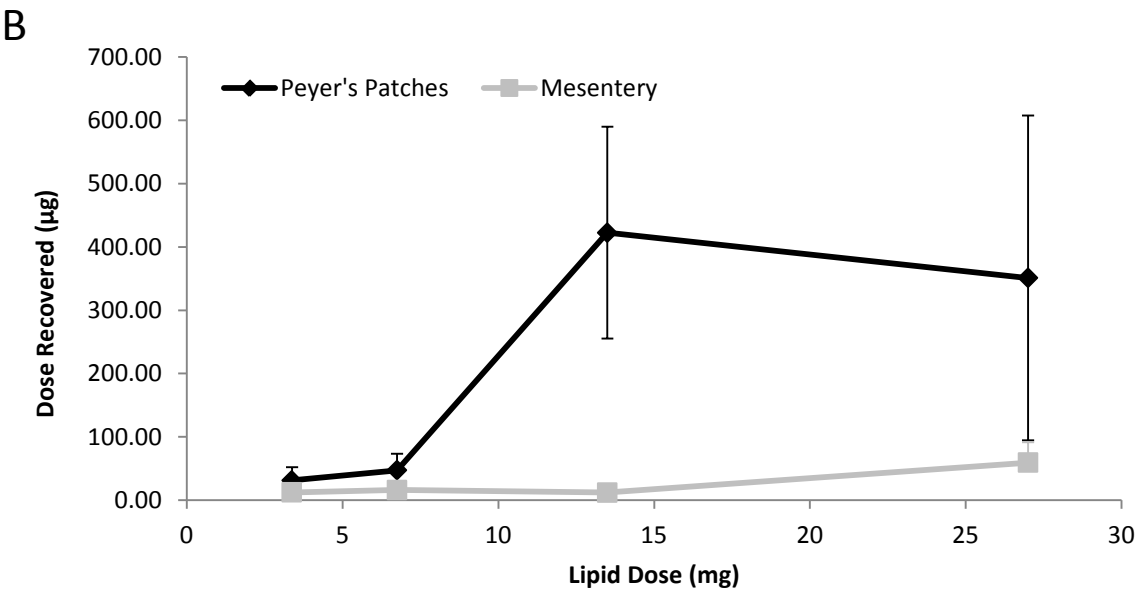
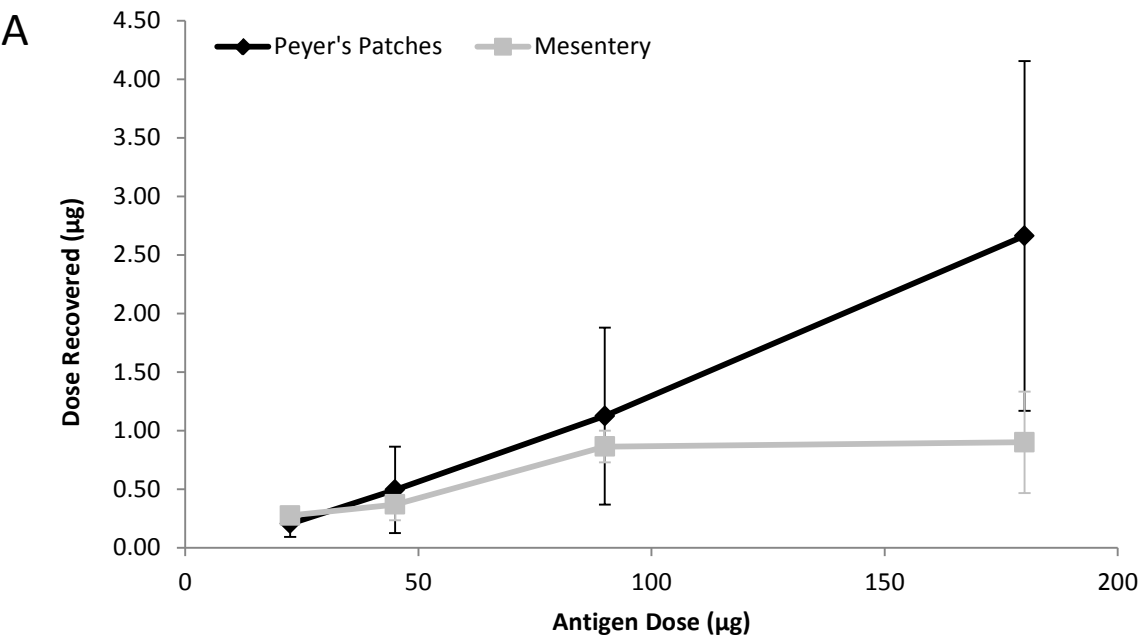


Figure 8.