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Formulation and manufacturing of lymphatic targeting liposomes using microfluidics.

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Key words: Liposomes; lymphatic targeting; avidin; biotin; protein delivery; microfluidics; vaccines

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Abstract

The lymphatics are a target for a range of therapeutic purposes, including cancer therapy and vaccination and both vesicle size and charge have been considered as factors controlling lymphatic targeting. Within this work, a range of liposomal formulations were investigated to develop a liposomal lymphatic targeting system. Initial screening of formulations considered the effect of charge, with neutral, cationic and anionic liposomes being considered. Biodistribution studies demonstrated that after intramuscular injection, anionic liposomes offered the most rapid clearance to the draining lymphatics with cationic liposomes forming a depot at the injection site. Anionic liposomes containing phosphatidylserine showed higher clearance to the lymphatics and this may be a results of preferential uptake by macrophages. In terms of vesicle size, smaller unilamellar vesicles gave high lymphatic targeting and 10-fold increases in concentration were achieved in dose escalation studies (up to 40 mg of lipids). Given that effective trafficking to the lymphatics was achieved, the next step was to enhance retention of the liposomes within the lymphatics, therefore this liposome formulation was combined with an avidin/biotin complex mechanism. The affinity of avidin for biotin allows biotinylated liposomes to complex in the presence of avidin. By pre-dosing with avidin, this biotin-avidin complex can be exploited to promote longer retention of the liposomes at the draining lymphatics. To load these small, biotinylated liposomes with protein, microfluidics manufacturing was used. Using microfluidics, protein could easily be incorporated in these small (~90 nm) biotinylated liposomes. Both liposome and protein retention at the local draining lymph nodes was demonstrated with the liposome-biotin-avidin system. These results demonstrate that microfluidics can be used to prepare protein-loaded liposomes that offer enhanced lymphatic targeting and retention of both the liposomes and entrapped antigen.

Introduction

The lymphatic system is an important component of the immune system and acts as a secondary circulation system to drain excess fluids, proteins and waste products from the extracellular space into the vascular system. The lymphatics have been exploited as a potential means of drug delivery as these channels can transport certain lipophilic compounds such as long-chain fatty acids, triglycerides, cholesterol esters, lipid soluble vitamins and some xenobiotics, including Dichlorodiphenyltrichloroethane (DDT) [1]. In particular, lymphatic capillaries play a vital role in the absorption and uptake of drugs into the lymphatic system and lymph nodes. The walls of lymphatics capillaries are made up of a single layer of endothelial cells that has numerous gaps and pores that permit passage of macromolecules into the capillary lumen when interstitial pressure exceeds the intraluminal lymphatic pressure [2]. The lymphatics are the primary conduit for the dissemination of metastases from many solid tumours; they play a pivotal role in the generation of immune responses and have been implicated in the pathogenesis of diseases including HIV and metastitial tuberculosis. For lymph resident diseases, lymphatic targeting of therapeutic drugs (e.g., antivirals, cytotoxics or immunomodulators) is therefore expected to provide an advantage.

Parenterally administrated vaccines mainly rely on the migration of APCs to the draining lymph nodes to present the processed cargo (e.g. antigens) to the T or B cells, which are found in large numbers within the lymph nodes [3, 4]. Therefore, promoting the delivery of vaccines into the lymphatics could improve cellular and humoural immune responses. Indeed, recent research has focused on the delivery of antigens or immunocomponents into the lymphatics as a means to enhance vaccine efficacy [5, 6].

To enhance lymphatic targeting, a range of design modifications have been applied to particulate delivery systems to enhance their drainage to the lymphatics including e.g. surface engineering [7], and optimisation of particle size and charge [8],[9]. In addition, strategies to enhance the retention (and avoid rapid clearance) of the liposomes after they reach the lymphatics have been considered. This involves administration of a dose of avidin subsequently followed by administration of biotin-bearing liposomes [10]. This results in the drainage of the avidin, followed by the biotin liposomes, to the lymph nodes. Due to the high binding affinity between biotin and avidin, upon draining to the lymphatics the avidin and biotin-liposomes will cross-link and become trapped within the lymph node [11]. Biotin, also known as vitamin H, is a small molecule (MW 244) that is present in very small amounts in all living cells and is critical for a number of biological processes. The valeric acid side chain of the biotin molecule can be derivatised in order to incorporate various reactive groups that are used to attach biotin to other molecules. The strong affinity of avidin for biotin allows biotin-

containing molecules in a complex mixture to be specifically bound to avidin. Avidin is a glycoprotein found in the egg white and tissues of birds, reptiles and amphibians. It contains four identical subunits having a combined mass of 67 to 68 kDa. Each subunit consists of 128 amino acids and binds one molecule of biotin, thus, a total of four biotin molecules can bind to a single avidin molecule. The extent of glycosylation on avidin is very high; carbohydrates account for about 10% of the total mass of the tetramer. Avidin has a basic isoelectric point (pI) of 10 to 10.5 and is stable over a wide range of pH and temperatures. Extensive chemical modification has little effect on the activity of avidin, making it especially useful for protein purification. However, because of its carbohydrate content and basic pI, avidin exhibits relatively high nonspecific binding properties. Avidin-biotin binding is the strongest known non-covalent interaction between a protein and ligand. The bond formation between biotin and avidin is very rapid, and once formed, is unaffected by extremes in pH, temperature, organic solvents and other denaturing agents. Due to the high binding affinity between biotin and avidin, pre-dosing and clearance of avidin to the lymphatics followed by injection of biotinylated liposomes (designed to drain to the lymphatics), could result in cross-linking of the liposomes and them subsequently becoming trapped and retained within the lymph node.

Therefore, this potential method of targeting of liposomes to lymphatics offers a range of advantages including the potential to both reduce non-specific organ toxicities and increase the chemotherapeutic dose to the most likely sites of loco-regional cancer metastasis. In addition, advanced lymphatic imaging tools will also improve cancer staging and reduce the need for destructive nodal dissections. Finally, targeting of liposomal sub-unit antigen systems may enhance their adjuvant activity. Thus, the aim of this study was to systematically investigate the influence of liposomal characteristics, including lipid composition, charge, particle size, and lipid dose for the lymphatic targeting, and then formulate liposomes, via microfluidics, that combine this targeting with a biotin-avidin complex strategy to enhance liposome retention in the local lymph nodes.

Methods

Materials

1,2-distearoyl-sn-glycero-3-phosphocholine (DSPC), 2-distearoyl-sn-glycero-3-phosphoethanolamine-N-methoxy-poly(ethylene glycol) 2000 (DSPE-PEG2000), 1,2-dioleoyl-3-trimethylammonium-propane (DOTAP), L-α-phosphatidylserine (PS), 1,2-dimyristoyl-sn-glycero-3-phospho-(1'-rac-glycerol) (DMPG) from Avanti lipids, Alabaster, AL, USA; Methanol and Chloroform (HPLC grade) from Thermo Fisher Scientific, Loughborough; Hydrogen peroxide, Cholesterol and Phosphate buffered saline tablets (PBS) from Sigma-Aldrich, Poole, Dorset, UK; Ultima Gold

scintillation fluid and ^{3}H-Cholesterol from Perkin Elmer, Waltham, MA; Foetal bovine serum (Heat-Inactivated) from Biosera, Leicestershire, UK; THP-1 cells were obtained from Roswell Park Memorial Institute; RPMI medium was purchased from PAA , Yeovil, UK; DilC 18 1,1'-dioctadecyl-3,3,3'3'-tetramethylindocarbocyanine perchlorate, from Molecular probes, Oregon, USA. H56 TB antigen was gifted by Statens Serum Institut (Copenhagen, Denmark).

Preparation of liposomes

Multilamellar vesicles (MLVs) were prepared by the established film technique first observed by Bangham, in the 1960s [12]. The lipids used throughout these studies were dissolved in chloroform: methanol (9:1 v/v) solution at the desired concentrations. The lipid solutions were placed in a 50 mL round-bottom spherical Quick-fit flask and the solvent was removed by rotary evaporation at about 37^{o}C. This yields a thin lipid film on the walls of the flask. The dry lipid film was hydrated by addition of 2 mL of phosphate buffer saline (PBS) and agitated vigorously until the thin lipid film was completely dissolved and transformed into a milky suspension. The hydration of the lipid film was maintained above the gel-liquid crystal transition temperature (T^{c}) of the phospholipids used.

To generate small unilamellar vesicles (SUV), MLV were disrupted via sonication using a probe sonicator (Soniprep 150). The tip of the sonication probe (diameter ~ 9.5 mm) was placed onto the surface of the 2 mL MLV mixture. The time required for sonication varied depending on the lipid composition (approximately 2-6 minutes). The SUV suspension was then centrifuged at 3,500 g for 10 minutes to remove any titanium debris released from the probe during sonication.

Preparation of protein loaded liposomes using microfluidics

To prepare liposomes, a Nanoassemblr™ Benchtop (Precision Nanosystems, Inc., Vancouver, Canada) was used with a 300 µm Staggered Herringbone Micromixer. The lipids at the appropriate ratio were dissolved in methanol along with DSPE-PEG 2000-Biotin (20 mol%) and H56 antigen was dissolved in PBS, 10 mM, pH 7.4 aqueous buffer. The flow rate ratio between the aqueous and solvent stream was 3:1 (aq:solvent ratio) and the total flow rate was 10 mL/min. Liposomes were purified using 750 kD tangential flow (TFF) column Spectra/Por® Dialysis membrane, Biotech CE Tubing, Spectrum Laboratories, USA). Liposomes washed for 10 diafiltration cycles using PBS buffer to remove solvent and unentrapped protein.

Determination of vesicle size, polydispersity and zeta potential of liposomal formulations

The z-average diameter and polydispersity (pdi) of liposomes was determined by dynamic light scattering using the photon correlation spectroscopy on a Malvern Zetasizer Nano-ZS (Malvern Instruments Ltd., UK); 100 µL of the sample was diluted with the hydration phase (e.g. PBS buffer solution) up to 1 mL and the vesicle size and pdi was measured at 25 °C. The zeta potential was also measured on Malvern Zetasizer Nano-ZS (Malvern Instruments Ltd., UK) at 25 °C. To measure the zeta potential, 100 µL of liposome suspension was diluted in 1 mL of its aqueous phase (1:300 v/v PBS).

Quantification of the protein or antigen loading within the formulations

Quantification of the protein loading within the liposomes was performed by reverse phase HPLC (RP-HPLC) using a UV detector (Agilent Technologies, Edinburgh, UK). A Jupiter 5 µ C18(2) column (Phenomenex, Cheshire, UK) pore size 300A was used as stationary phase. For the preparation of the standards and samples, protein alone or liposomes loaded with protein, were diluted in 50% PBS/IPA (1:1 v/v). A gradient elution method was followed where both mobile phases contain the same solvents in different proportions (Mobile phase A: 90% H20, 10% acetonitrile and 0.1% TFA; Mobile phase B: 70% acetonitrile, 30% H20 and 0.1% TFA). The column temperature was set at 60°C, the injection volume was 50 µL and the flow rate 1 mL/min.

Cryo transmission electron microscopy of liposomes

Liposome suspensions were diluted 1:10 with dilution buffer at room temperature immediately prior to vitrification. Samples were preserved by vitrification and supported on holey carbon films on 400-mesh copper grids. Vitrified samples were prepared by applying an 8 µL drop of sample suspension to a cleaned grid, blotting away with filter paper, and immediately plunging into liquid 30% propane/ethane refrigerated in liquid nitrogen. Grids were stored under liquid nitrogen until transferred to the electron microscope for imaging. Electron microscopy was performed using a JEOL 2010 FEG Transmission Electron Microscope (TEM) operating at 200 kV at Warwick University, UK. Vitreous ice grids were transferred into the electron microscope using a cryostage that maintains the grids at a temperature below −170°C. Images of each grid were acquired at multiple scales to assess the overall distribution of the specimen. After identifying potentially suitable target areas for imaging at lower magnifications, pairs of high magnification images were acquired using Image J software. General particle selection was based on the following criteria: particles were not selected if they were over the carbon film surrounding the hole or touching the edge of the carbon film.

Liposome stability in simulated biological media

Liposome systems were immersed in PBS buffer and/or in FBS, in a 50% v/v mixture with PBS buffer, in order to mimic *in vivo* conditions. Measurements of particle size and zeta potential of the systems was carried out via dynamic light scattering using a ZetaPlus analyser (Brookhaven Instrument Corporation). Upon the addition of each liposome batch in PBS buffer to an equal volume of FBS/PBS (50% v/v), samples were incubated in a water bath at 37 °C over 24 hours. At various intervals across this time period, the samples were centrifuged (Beckman Coulter - 125,000×g, 4 °C, 45 mins), prior to measurements of particle size and zeta potential as previously conducted.

***In vitro* studies**

Cell uptake studies were performed using the human monocyte cell line THP-1 as previously described [13, 14]. THP-1 cells were resuspended in fresh medium (RPMI 1640 + 10% v/v FCS) at a density of 5×10^5 cells/mL and stimulated for 48 h with 100 nM dihydroxyvitamin D3 to differentiate cells. Prior to use, cells were resuspended at 2×10^6 cells/mL in fresh RPMI with 10% (v/v) FCS. Liposomes (1 mg/mL) were labelled with 1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate) (DilC) (0.1 mol %) by inclusion of the lipid (dissolved in solvent) in the solvent evaporation stage of liposome production (as described above). To ensure that all formulations incorporated the DilC fluorophore equally, the fluorescence was measured using a fluorimeter. Fluorescently labelled liposomes were diluted to a concentration of 10 µg/mL in RPMI, mixed with cells (1:1) in tissue culture plates, and co-cultured at 37 °C in 5% CO_2. At various time-points, 200 µL of co-culture was removed and mixed with 200 µL ice-cold RPMI prior to immediate analysis. Association of fluorescent liposomes with THP-1 macrophages was analysed using non-fixed cells via flow cytometry using a Beckman-Coulter FC500 cytometer (High Wycombe, UK). For each sample a minimum of 10 000 events was examined.

Biostribution studies

All *in vivo* studies were conducted under the regulations of the Directive 2010/63/EU. All protocols have been subject to ethical review and were carried out in a designated establishment. Inbred BR/NMRI strain of mice used for research in biodistribution studies and were obtained from Aston Biomedical facility, Aston University, UK. BR/NMRI mice were used for *in vivo* biodistribution studies in order to study the movement of liposomes from the site of injection (SOI). Mice were housed under conventional conditions (22 °C, 55 % humidity, 12 h day/night cycle) in their experimental groups (8 mice/cage) and were given a standard diet ad libitum. All mice were female and purchased at 6-9 weeks of age. Groups of 3 mice were used to study biodistribution of liposomal formulations

for each time point and five time points were selected for termination of the mice which were 1 h, 1, 2, 4 and 8 days after the injection. Mice were injected 50 µL intramuscularly to the left quadriceps muscles. Muscle from the site of injection (left quadriceps) in case of intramuscular injection, spleen, popliteal lymph nodes (PLN) and other relevant lymph nodes were collected after termination of each mouse by cervical dislocation at relevant time points during the study. Each tissue sample was weighed in a plastic scintillation vial. To solubilise the tissues, 2 mL of 10 M NaOH were added and the samples incubated at 60 °C for 5 h. After all tissues were solubilised, 200 µL hydrogen peroxide was added and kept for 2 h at 60 °C to bleach the organs and solution completely.

3H and 125I has energy overlap at 0-18.6 keV (3H) and 0-80 keV (125I) and this leads to an over-spill due to iodine in tritium region. To eliminate this over-spill, a calibration curve was prepared at a plot of the cpm values derived from the scintillation counter due to 125I (x-axis) against the cpm values derived from the scintillation counter due to 3H (y-axis) was made and the line of best fit and equation derived for samples below 50,000 cpm (~2% of the dose or less) and those above 50,000 cpm (~2% of the dose or more). These two equations were used to eliminate the effective interference that the 125I would have on the 3H values determined by scintillation counting. 10 mL Ultima Gold™ scintillation fluid added to each sample and presence of 3H and 125I was quantified using a method for 3H-125I detection protocol using a TriCarb 2810 Liquid Scintillation Counter (Packard). Simultaneously, triplicate samples of the original dose in another plastic scintillation vials were made and measured for presence of 3H and 125I to calculate % original dose of liposomes (3H) and antigen (125I).

Immunisation Studies

Female 6-8 week-old C57BL/6 mice were used to study immunisation of above formulations. 5 mice per group were injected i.m (50 µL) into the left quadriceps with formulations prepared on the day of injection. Each mouse received a total of 3 injections on day 1, 14 and 28. The mice were vaccinated as follows: Group 1 was the control group, this group was vaccinated with 0.1 mg/mL H56 antigen; group 2 was vaccinated with H56 loaded DSPC:Cholesterol:PS; group 3 was vaccinated with H56 loaded DSPC:Cholesterol:PS:PEG2000 biotin (20 mole%) liposomes, and finally group 4 was vaccinated with the same formulation as group 3 plus avidin, which was injected on the same quadriceps 2 hours prior the main immunisation at a concentration of 200 µg per dose (50 µL).

Antibodies were detected in the serum of tail-bled mice. Blood was and placed into Eppendorfs coated with 1% w/v heparin. All tubes were centrifuged (12,000 rpm, 10 minutes) and the supernatant from each tube was placed in a new Eppendorf and frozen (- 20 °C) until use. Serum was

consequently 20-fold diluted in PBS if the volume ratio of red blood cells:sera is assumed to be 1:1. To detect antibodies in serum, standard enzyme-linked immunosorbent assays (ELISA) were used. ELISA pates (MaxiSorp) were coated overnight at 4 °C with 100 µL H56 in carbonate buffer (0.5 µg/well). Plates were washed three times to remove unbound antigen; the wash buffer used throughout was PBST (0.2% Tween 20, in PBS (10 mM)). Plates were blocked for 1.5 h at room temperature with 2% BSA (200 µL/ plate). After blocking, the plates were washed three times with PBST. 11 µL of the serum was added into the first well containing 100 µL of 1% BSA in PBS and mixed up and down with the pipette. Serial dilutions (1/10) were done by transferring 11 µL from the first well to the next one and so on 7 – 12 times. Lastly 11 µL of diluted serum was removed from the last wells so that the total volume of serially diluted serum in all wells was 100 µL. Each sample was investigated in duplicate. Plates were incubated at room temperature for 2 h followed by three washes with PBST and addition of 100 µL of isotype specific immunoglobulin diluted 1:5000 (IgG1) or 1: 20000 (IgG2c) in 1% BSA. After 1 h at room temperature all plates were washed again five times and 100 µL of TMB substrate added per well. After 20 mins of incubation in dark with TMB substrate, reaction was stopped by adding 100 µL/well of 0.2 M sulfuric acid. Absorbance were measured at 450 nm. The results were expressed as the log10 value of the reciprocal of the end point dilution which gave an optical density (O.D) of 0.2 or above.

Spleens and popliteal lymph nodes from each mouse were isolated and processed on day 49. Individual spleens were placed in 15 mL falcon tubes containing 2 mL of PBS and kept ice-cold whereas the lymph nodes from each group were pooled together. Spleens and lymph nodes were forced through a metal mesh using the bottom part of a 3 mL plastic syringe and transferred back to a falcon tube filled with 12 mL of PBS. Samples were washed twice via centrifugation at 1800 rpm for 5 min at 4°C. Cells were resuspended in 1.8 mL or 0.5 mL of complete RPMI (RPMI supplemented with HEPES, penicillin-streptomycin, L-glutamine, sodium pyruvate, non-essential amino acids, mercaptoethanol and 10% heat inactivated FCS) for spleens and lymph nodes respectively. Cells were counted and diluted in complete RPMI to a final concentration of 2×10^6 cells/mL. Cells were counted using the tryphan blue exclusion assay. Briefly, cells were dyed with tryphan blue in a 1:10 ratio (10 µL cells: 90 µL tryphan blue). Subsequently, 100 µL were placed on a haemocytometer and cells were counted.

Once the cells were diluted to the same concentration, 100 µL of this diluted cells were plated on a Nunclon 96-well round bottom (Fisher scientific, Loughborough, UK). Cells were stimulated with either 100 µL of ConA (5 µg/mL) which serves as a positive control since it stimulates the production of cytokines, RPMI media as a negative control, or H56 antigen (5 µg/mL) as the investigated

antigen. Splenocytes and lymph nodes from immunised mice were incubated at 37°C, 5 % CO_2 and 95 % humidity for 72 hours. After 3 days of incubation, supernatants were harvested (approximately 160 µL) and stored at -20°C for further processing. Supernatants from restimulated splenocytes and lymph nodes were analysed using a sandwich ELISA protocol for the production of cytokines IL-17 and IFN-ɣ. Plates were coated with the specific capture antibody diluted in carbonate buffer, either purified anti mouse IL-17 (1:500 dilution) or IFN-ɣ (1:1000 dilution) overnight at 4°C. Next day plates were emptied and blocked for 1.5 – 2 hours at room temperature with 200 µL/well PBS containing 2% milk powder. Plates were washed and samples and standards were diluted in 2% BSA in PBS and incubated at room temperature for 2 hours (total volume in all wells was 100 µL). Plates were washed 3 times and biotin conjugate was diluted in 1% BSA in PBS (IL-17 1:2000, IFN-ɣ 1:5000) and 100 µl were added on each well. Plates were incubated for an hour at room temperature followed by washing and 30 min incubation with 100 µL/well of HRP-streptavidin in 1% BSA (1:5000). Plates were washed and TMB substrate (room temperature) was applied to the plates 100 µL/well. After approximately 15 minutes the reaction was stopped by adding 100 µL/well 0.2 M sulfuric acid.

Statistical analysis

Statistical analysis was carried out by one-way ANOVA (analysis of variance) at a significance level of 0.05. All statistical analyses were performed using Graph Pad Prism software (Version 6.0 for Windows). Statistically significant differences are noted ($P < 0.05$). Each experiment was carried out in triplicate with the average mean result recorded ± the standard deviation of this set of results (represented as error bars).

Results

The choice of anionic lipid within the liposomes impacts on the distribution to the lymphatics and may be influenced by phagocytic uptake

Given that previous research has suggested the neutral liposomes clear more effectively to the draining lymphatics [9, 15, 16], we initially tested four liposomes of different charges: neutral - DSPC:Cholesterol, anionic - DSPC:Chol:PS and DSPC:Chol:DMPG and cationic - DSPC:Chol:DOTAP. All liposomes were approximately 100 nm in size and PDI values of 0.2 with their zeta potential being near neutral (DSPC:Cholesterol), anionic (with the addition of PS or DMPG) or cationic (with the inclusion of DOTAP) respectively (Figure 1 A). These four formulations were injected intramuscularly and clearance from the injection site monitored. From Figure 1B, we see the cationic DOTAP formulation shows high retention at the injection site with approximately 40% retained after 192 h (4 days). The neutral (DSPC:Chol) and anionic (PS and DMPG based formulations) showed faster

clearance with the DSPC:Chol:PS formulation showing the fastest clearance; only 23 % of the liposome dose remaining after 24 h. These clearance profiles translated to appearance at the draining local lymph node (popliteal lymph node; POP), with DSPC:Chol:PS liposomes showing the highest accumulation (approximately 30% at 24 h) within the lymph node (Figure 1C). When considering the interaction of these formulations with the biological environment, the cationic liposomes rapidly aggregate in the presence of simulated biological media (50% v/v foetal bovine serum (FBS), whilst there is no notable aggregation occurring with the neutral nor anionic formulations (Figure 1D). The FBS media consisted of 50% FBS and 50% PBS buffer (v/v) as blood plasma represents ~50% of total blood volume, thus mimicking the physiological conditions that the liposomal drug may encounter upon injection. This suggests that the cationic liposomes will aggregate upon injection and form a depot at the injection site. However, this does not explain the differences in clearance of the neutral and anionic formulations. The neutral and both anionic liposome formulation are similar in size and, due to the presence of cholesterol within their formulation, have similar low transition temperatures.

A)

Formulations	Lipid ratio (µmoles)	Particle size (nm)	pdi	Zeta Potential (mV)
DSPC:Cholesterol	6:4	101 ± 1	0.17	-2 ± 0.6
DSPC:Cholesterol:DMPG	6:4:2.5	92 ± 3	0.23	-55 ± 3
DSPC:Cholesterol:PS	6:4:2.5	90 ± 7	0.22	-66 ± 4
DSPC:Cholesterol:DOTAP	6:4:2.5	110 ± 4	0.18	62 ± 5

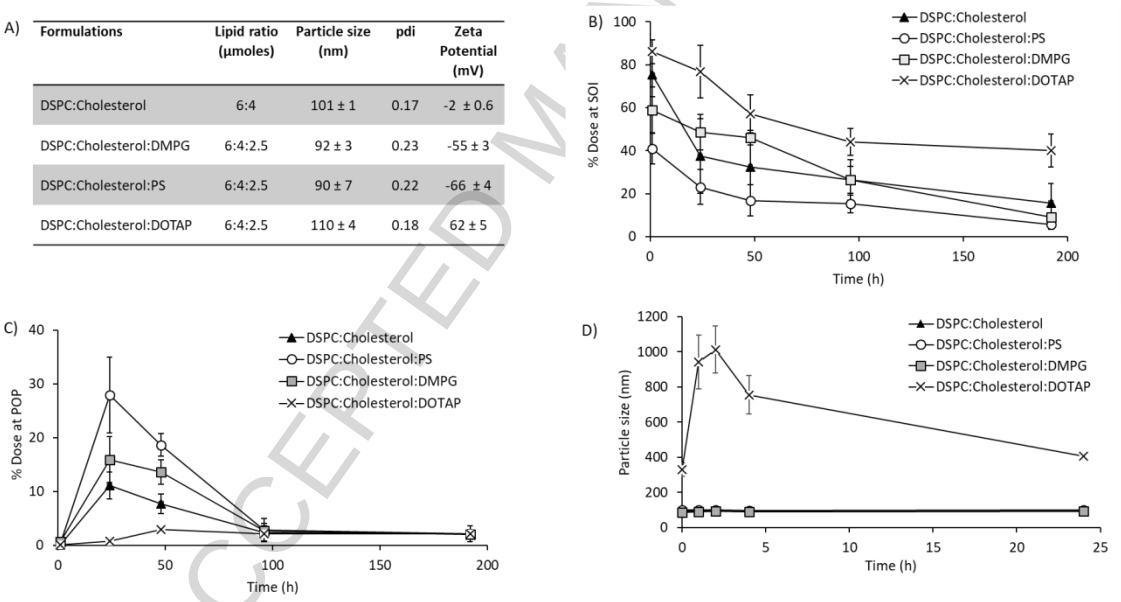

Figure 1. The effect of charge on liposome biodistribution. Mice were injected intramuscularly with 4 formulations, as shown in (A). The rate of clearance from the injection site (B) and the popliteal lymph node (POP) (C) are shown as a percentage of injected dose. The degree of aggregation of these formulations when exposed to simulated interstitial fluid is shown in D. Results are expressed of the mean ± SD of 3 mice per group.

Given that clearance of the liposomes from the injection site may also result from phagocytic cell uptake, we compared the cellular uptake of the neutral and anionic liposome formulations using the human macrophage cell line THP-1 [14] (Figure 2). The uptake studies were performed *in vitro* using fluorescence-labelled liposomes co-cultured with THP-1 derived macrophages at a final lipid

concentration of 10 µg/mL. The proportion of macrophages associated with fluorescent liposomes, and the relative amount of fluorescence associated, was quantified using flow cytometry. Figure 2 shows that liposomes containing PS are more rapidly taken up by the macrophages that their DMPG and neutral liposome counterparts. For PS liposomes, around 80% of the cells were associated with the liposomes compared to DMPG liposomes which had approximately 40% uptake (Figure 2A); this may be due to the presence of PS serving as a recognition signal for phagocytosis by macrophages [17]. Indeed the rapid uptake of the PS liposomes could be negated by PEGylating the DSPC:Chol:PS liposomes. With this formulation, the macrophages showed only 20 % of cells association with fluorescence (Figure 2B). This proves that uptake of these liposomes to cells is not only dependent on the general charge of the liposomes but also based on the anionic lipid adopted within the formulation with PS liposomes showing selective uptake.

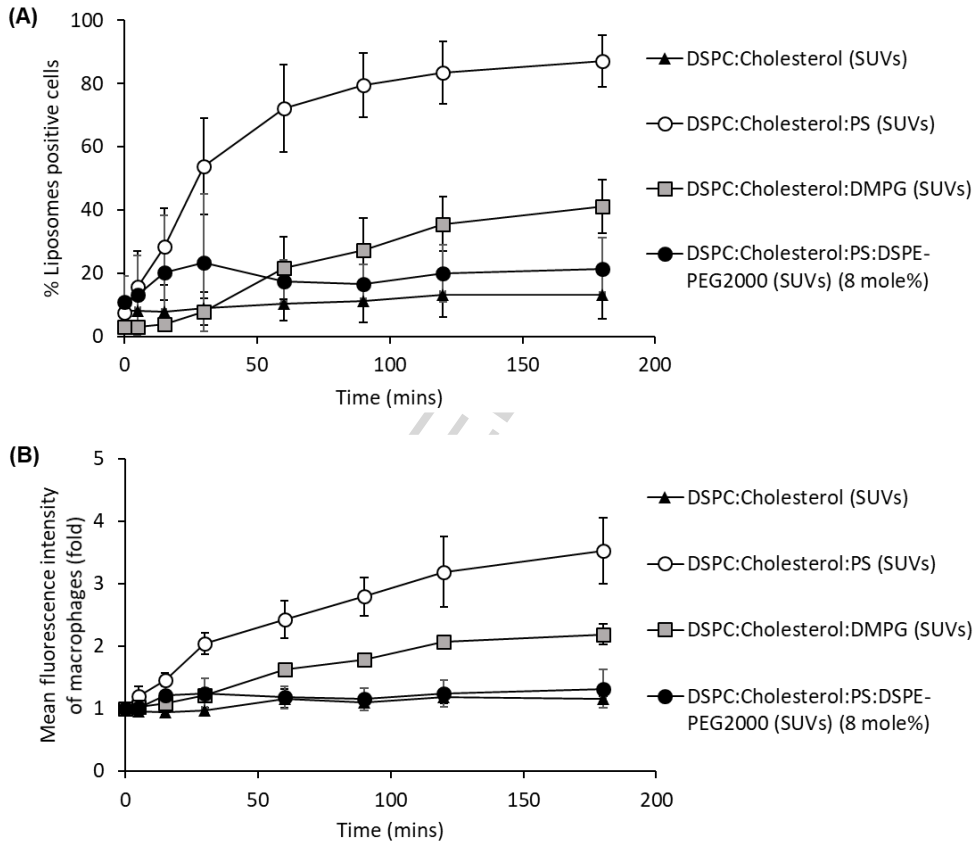

Figure 2. The effect of formulation on liposome update by macrophages. Association of liposomes with THP-1 (macrophages) cells was investigated by flow cytometry. (A) Percentage of THP-1 cells positive for fluorescence surface bounded or internalized) with macrophages at 0 mins and 180 mins. (B) Fold increase of mean fluorescent intensity of THP-1 cells co-cultured with above liposome formulations. Results denote mean ± SD of 3 independent experiments.

The size of anionic liposomes also influences liposome clearance from the injection site and movement to the lymphatics

Several studies have suggested that smaller vesicles can clear to the draining lymphatics (e.g. [18-21]), therefore we also tested and confirmed this with the DSPC:Chol:PS formulation formulated as small (<100 nm; Figure 3A) and large (approx. 900 nm; Figure 3B) vesicles. The results in Figure 3C and D, show that the smaller liposomes clear more rapidly from the injection site and move to the draining lymph node with only low levels (< 5 %) of the MLV being detected at the popliteal lymph node (Figure 3D). Interestingly, uptake by macrophages was also higher for the SUV compared to the MLV (Figure 3E and F). So it is not clear if the difference in clearance is due to drainage of the liposomes or their phagocytic uptake and maybe an accumulation of both.

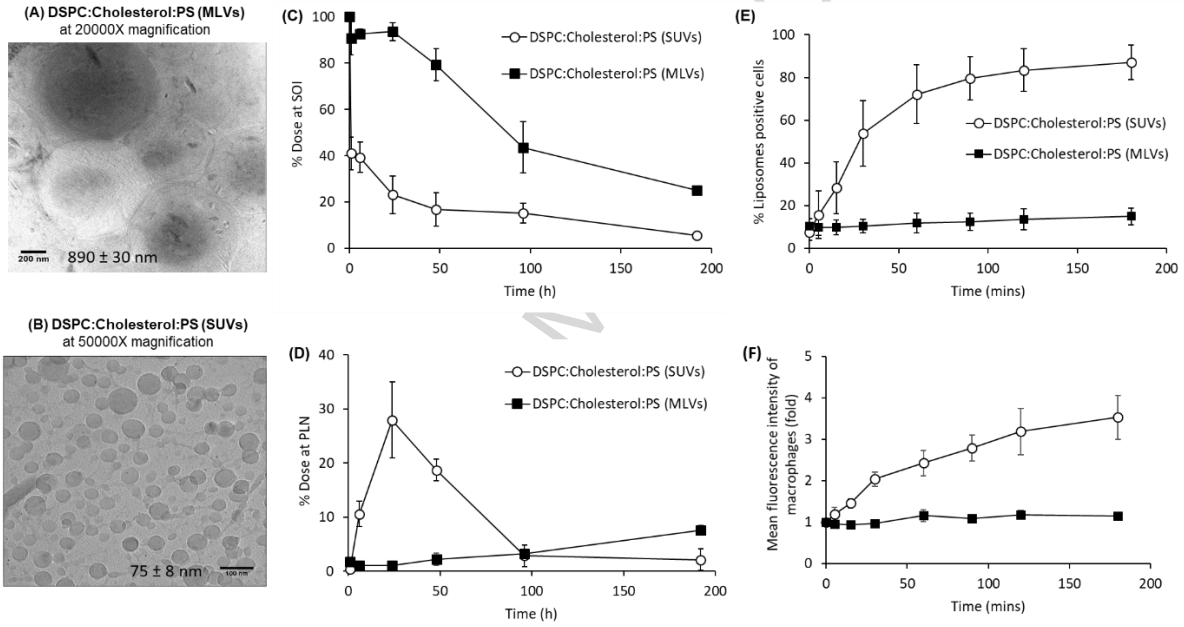

Figure 3 The effect of liposome size on the clearance of liposomes from the injection site to the local draining lymph node. DSPC:Chol:PS liposomes were formulated as MLV (A) and SUV (B). Mice were intramuscular (i.m.) injected with SUV and MLV liposomes and the liposomes at the injection site (C) and popliteal lymph node (PLN) (D) are shown. (E) Percentage of THP-1 cells positive for fluorescence surface bounded or internalized) with macrophages at 0 mins and 180 mins. (F) Fold increase of mean fluorescent intensity of THP-1 cells co-cultured with above liposome formulations. Results are expressed of the mean ± SD of 3 mice per group.

A dose-dependency of the accumulation of liposomes in regional lymph nodes

To study the effect of anionic lipid dose on lymphatic targeting of liposomes, liposomes with increasing amount of lipid dose of PS (i.e. 48, 240 and 480 µg/dose) were injected intramuscularly.

The recovery of liposomes (expressed as dose of PS) at the site of injection is shown in figure 4A; approximately 40 to 50 % of PS dose was recovered at injection site at 1 h. All formulations were cleared from site of injection within 48 h (Fig 4A). The increasing trend shows no saturation of the lymphatic absorption process was observed over the dose range investigated until 24 h. However, lipids were seen to be cleared from lymph nodes after 48 h of injection; 12 µg dropped to 8 µg at 24 and 48 h respectively when 48 µg of PS was injected (Fig 4B). A similar trend was seen in case of 5-fold and 10-fold doses of PS (Figure 4C) when expressed as percentage dose, demonstrating that at the concentrations tested, saturation did not occur. This suggests that the clearance from the injection site and movement to the lymph node may be both a physical and cellular process.

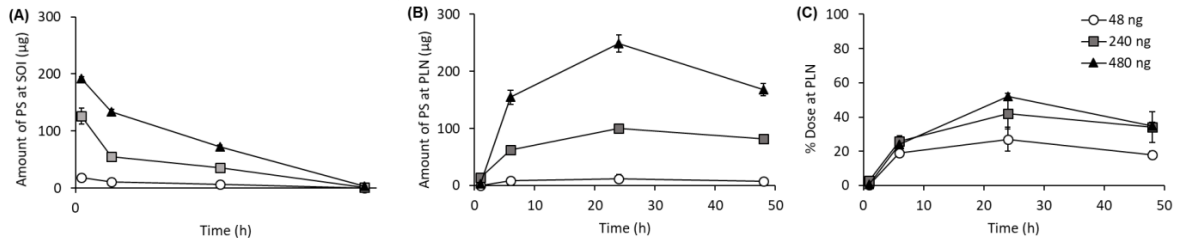

Figure 4. The effect of lipid dose on lymphatic uptake in mice following intramuscular injection of liposome formulations. Increasing doses of DSPC:Chol:PS liposomes were injected and the amount of liposomes at the injection site measured and shown as (A) Amount of PS (µg) at Injection site, (B) Amount of PS (µg) at Popliteal lymph node (PLN), (C) Percent dose of DSPC:Cholesterol:PS liposomes at three different escalated doses at popliteal lymph nodes. Results are expressed of the mean ± SD of 3 mice per group

Exploiting a biotin-avidin complex enhances retention of liposomes at the draining lymph node

Given we had identified a liposome formulation that offered rapid clearance to the local lymphatics, the next stage was to develop the formulation further to offer enhanced retention at the lymph node. To achieve this, an avidin-biotin complexing process was investigated [11]. To allow us to effectively load the liposomes being formulated we also adopted microfluidics as the manufacturing process. We have recently demonstrated that microfluidics offers the potential to prepare small unilamellar vesicles with high protein loading [22]. Therefore, two liposomes formulations were prepared using Nanoassemblr based on DSPC:Chol:PS:DSPE-PEG2000 but with one formulation having biotin conjugated to the PEG. Initially both liposome formulations were prepared without protein and both formulations were similar in size and charge (100 - 120 nm; 0.19 – 0.22 PDI; zeta potential -16 to -20 mV; Figure 5). Mice were intramuscularly injected with either non-biotinylated PS-PEG 2000 liposomes (DSPC:Chol:PS-PEG2000), biotinylated liposomes without pre-dosing of

avidin (DSPC:Chol:PS:PEG2000-biotin) or biotinylated liposomes with pre-dosing of avidin 2 h prior to liposome administration (DSPC:Chol:PS:PEG2000-biotin-avidin) (Figure 5).

Formulation	Size (nm)	PDI	Zeta Potential (mV)
DSPC:Chol:PS:PEG2000	101 ± 5	0.20 ± 0.01	-16 ± 5
DSPC:Chol:PS:PEG2000-biotin	119 ± 3	0.10 ± 0.01	-20 ± 4
DSPC:Chol:PS:PEG2000-biotin-avidin	119 ± 3	0.10 ± 0.01	-20 ± 4

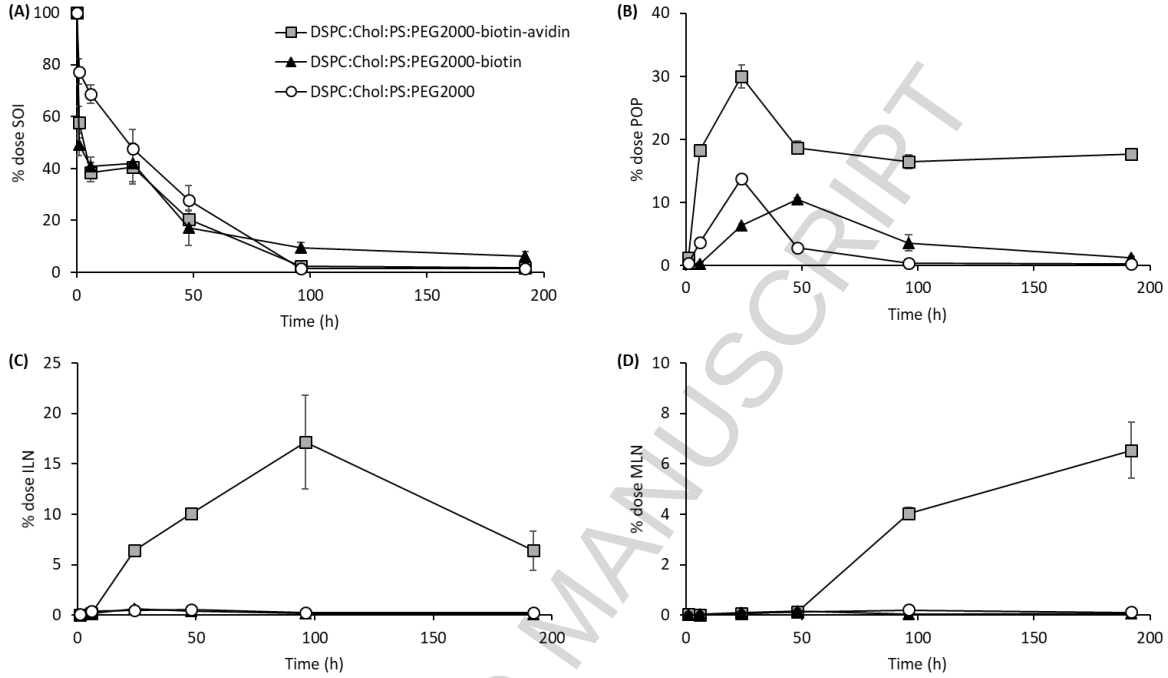

Figure 5. The effect of exploiting a biotin-avidin complex system for lymphatic targeting.. Mice were intramuscularly injected with either DSPC:Chol:PS:PEG2000 (6:4:2.5 µmoles; 2 mole% PEG) or DSPC:Chol:PS:PEG2000-biotin (6:4:2.5 µmoles; 2mole% PEG-biotin). Two groups of mice received the biotin-coated liposomes, one without pre-dosing of avidin (DSPC:Chol:PS:PEG2000-biotin) and one group receiving a pre-dose of avidin (DSPC:Chol:PS:PEG200-biotin-avidin). The percentage (%) injected dose of biotinylated liposomes in mice following intramuscular injection A: Injection site, B: Popliteal lymph node (POP), C: Inguinal lymph node (ILN) and D: Mesenteric lymph node (MLN). Results are expressed of the mean ± SD of 3 mice per group.

In terms of clearance from the injection site, all three formulations rapidly cleared; approximately 50 % of the initial dose remained at the injection site after 24 h and approximately 20 % at 48 h suggesting that the presence of biotin on the liposome formulation did not influence clearance from the injection site (Figure 5A). However, upon clearing the injection site, mice pre-dosed with avidin and subsequently injected with biotinylated liposomes (DSPC:Chol:PS:PEG2000-biotin-avidin) showed higher lymph node retention (Figure 5B to D) with approximately 30 % after 24 h ($P < 0.05$) at the popliteal lymph node compared to the two other groups (Figure 5B). Furthermore, the avidin-biotinylated liposome combination showed prolong retention at the popliteal lymph node with

approximately 20 % of the dose remaining after 8 days whereas non-biotinylated liposomes showed less than 1% of dose at lymph node on day 8 (Figure 5B) suggesting the formation of a biotin-avidin complex within the lymph node and increased accumulation and retention at draining lymph nodes [10]. The use of the biotin-avidin complex system also promoted higher retention at other nearby lymph nodes, especially the inguinal lymph node which showed approximate 15 % ($P < 0.05$) uptake at 96 h when pre-dosed with avidin, yet liposomes without biotin or avidin showed no uptake in any secondary draining lymph nodes (Figure 5C and 5D).

Biotinylated liposomes offer enhanced trafficking and retention of liposomes and their entrapped moiety to local and secondary lymph nodes.

Using a dual-radiolabelling technique, the biodistribution of liposomes and antigen was studied in mice (Figure 6). Two liposome formulations were prepared entrapping the H56 protein antigen (Table 1) with the liposomes being 90 to 115 nm in size and protein loading being approximately 20% for the PS liposomes and 10% for the PEGylated PS liposomes. Figure 6A shows the draining of PS liposomes along with the antigen from site of injection. Only 10 % of liposomes and antigen was present at site of injection at 48 h. These liposomes then move to the popliteal lymph node along with the entrapped antigen (Figure 6B), with no detectable levels of liposomes or protein being noted at the inguinal or mesenteric lymph node (Figure 6C and D respectively). In comparison, the combination of pre-dosing with avidin and subsequent injection of biotinylated liposomes resulted in a faster clearance of the liposomes from the injection site with only 23 % of liposomes and 13 % of antigen respectively being present at 6 h (Figure 6E). In parallel to this clearance, rapid accumulation of the liposomes and antigen is seen at the popliteal lymph node with approximately 35 % of both the liposomes and antigen locating at this lymph node after only 6 h and 13% of the antigen still being present after 4 days (Figure 6F). In the case of the inguinal and mesenteric lymph nodes, notable levels (up to 25%) of the liposomes and antigen accumulated (Figure 6G and H respectively). This is compared to no liposome or antigen being detected at either site when the avidin-biotin complexation was not used. These results demonstrate that the biotin-liposome/avidin method increasing the deposition of liposomes in lymph nodes delivers their entrapped moiety to the draining lymph nodes and that this targeting and retention was also measured in secondary lymph nodes such as inguinal or mesenteric lymph node.

Table 1: Physicochemical characteristics (particle size, PDI and ZP) and loading efficiency of the vaccine formulations prepared for the immunisation study. All vaccine formulations were loaded

with a final H56 concentration of 0.1 mg/mL. Non-encapsulated antigen was removed by TFF in order to have the same antigen concentration in all the vaccines.

Formulations	Lipid ratio (µmoles)	Particle size (nm)	PDI	Zeta Potential (mV)	Antigen loading (%)
DSPC:Cholesterol:PS	6:4:2.5	113.3 ± 2.3	0.15 ± 0.02	-41.1 ± 4.6	18.7 ± 4.3
DSPC:Cholesterol:PS:DSPE-PEG 2000 biotin (20 mole %)	6:4:2.5 + 20 mole%	87.5 ± 6.7	0.24 ± 0.02	-11.5 ± 1.9	9.9 ± 3.2

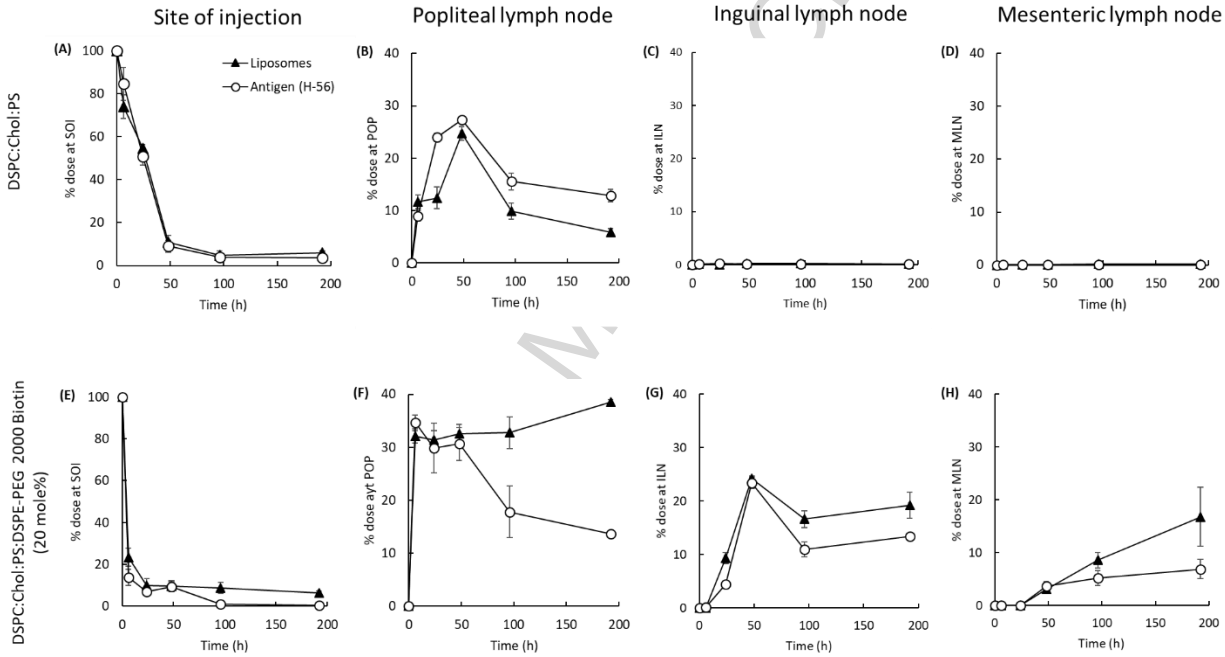

Figure 6. Biodistribution of lymphatic targeted liposomes and antigen (5 µg/dose H-56) in mice. Two liposome formulations were tested (avidin was intramuscularly injected 2 hours prior to injection of the main formulation). Liposomes and antigen dose was monitored at the site of injection (A & E), popliteal lymph node (B & F), Inguinal lymph node (C & G) and mesenteric lymph node (D & H) were isolated and counted for % injected dose of DSPC:Chol:PS and DSPC:Chol:PS:DSPE-PEG 2000 Biotin (20 mole%) formulations respectively. Results represent the mean ± SD of three replicate batches (n = 3).

Enhanced retention at the lymph node did not impact on immune responses

To test if this enhanced delivery and retention of the liposomes and their entrapped protein generated immune responses, immune responses against the entrapped antigen were also

evaluated (Figures S1 and S2). The impact of lymphatic retention was tested by comparing mice which did and did not receive a pre-dose of avidin prior to receiving an intramuscular injection of biotinylated liposomes incorporating the H56 protein antigen. Comparing across the liposome formulations, there is no significant difference in antibody (Figure S1) or cytokine (Figure S2) responses in both the spleen and lymph nodes. This suggests that re-directing and retaining liposomes that do not contain immunostimulatory agents does not promote notable immune responses.

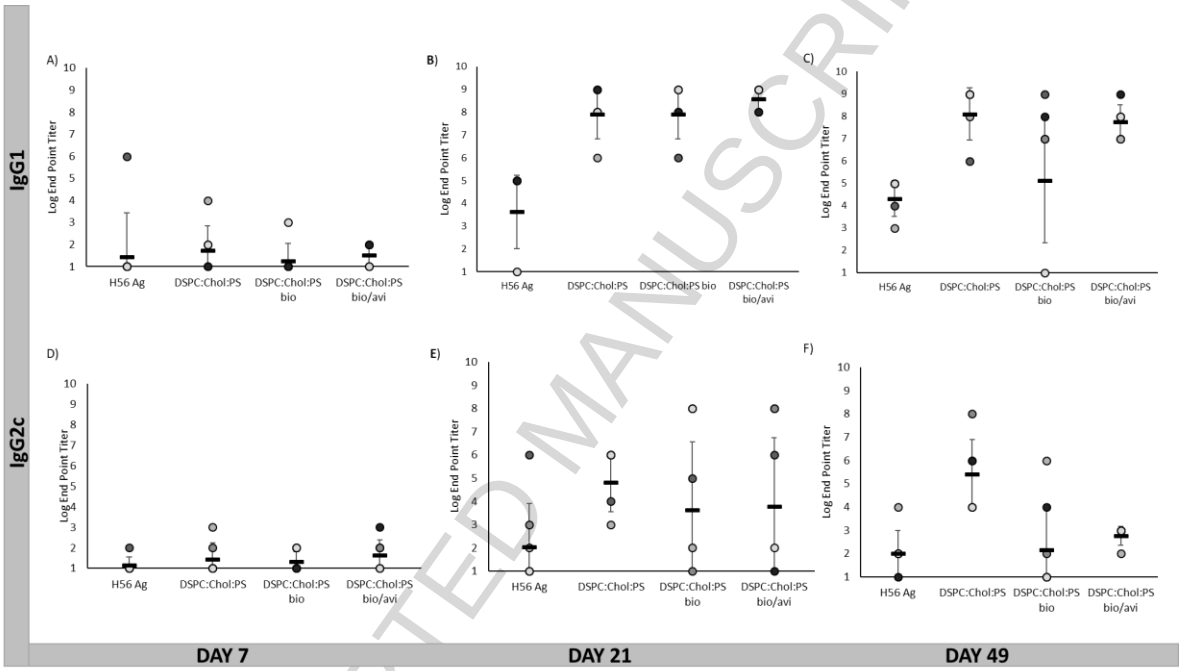

Figure S1. Antigen-specific (A) IgG1 and (B) IgG2c responses in mice immunised with liposomes with and without the biotin-avidin complex formulation. C57BL/6 mice were intramuscularly immunised with the liposomes incorporating 5 µg H56 and humoural responses were analysed in blood. H56-specific IgG1 (A, B, C) and IgG2c (D, E, F) serum responses detected by ELISA on sera collected (A, D) 7, (B, E) 21 and (C, F) 49 days after i.m. immunization. Antibody titres were expressed as the reciprocal log of the highest dilution with an OD value ≥0.2 after background subtraction.

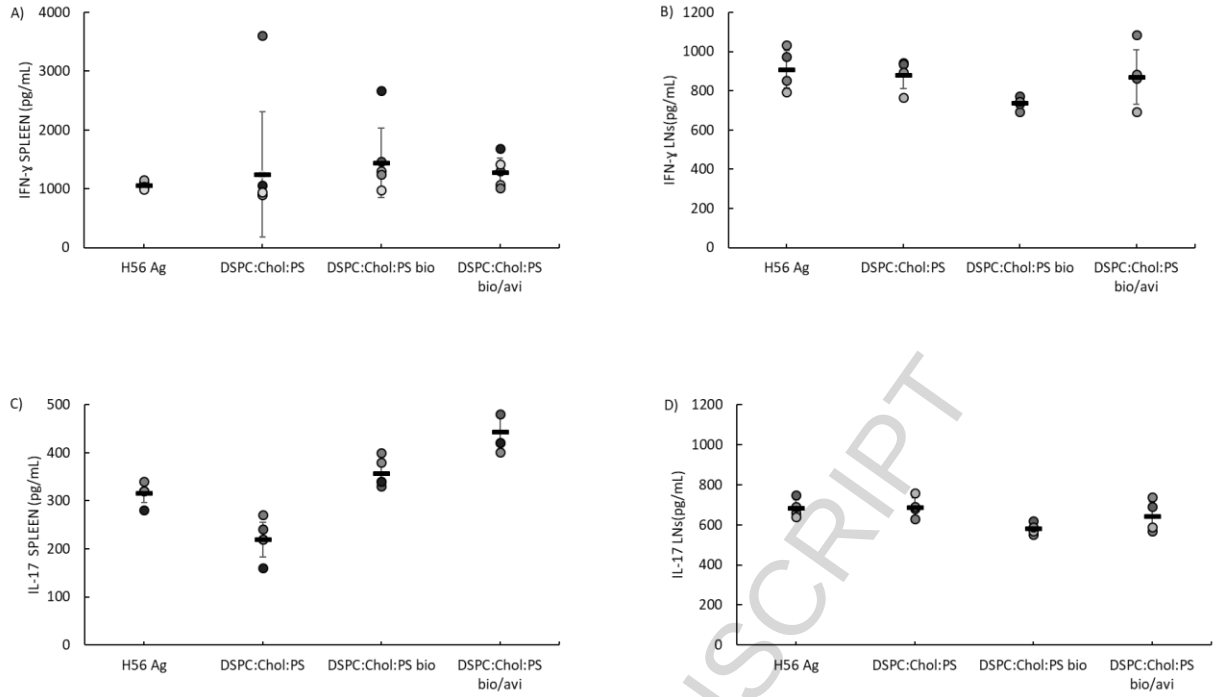

Figure S2. Cytokine production in splenocyte and lymph node culture supernatants (IL-17 and IFN-ɣ) in mice immunised with liposomes with and without the biotin-avidin complex system. C57BL/6 mice were intramuscularly immunised with 5 µg H56 incorporated in the various liposome formulations and spleens were collected 3 weeks after the last immunization. Levels of IFN-ɣ in the spleen (A), popliteal lymph nodes (B), and IL-17 in the spleen (C) and lymph nodes (D) were measured. Values, expressed as picograms per milliliter, are reported as the mean value ± SD of H56-stimulated of five animals per group.

Discussion

It has been extensively studied and published that lipid composition and charge affect liposomal biodistribution particularly, lymphatic uptake and targeting [23-25]. Building on this, from our studies we are able to confirm that in addition to the formulation of small negatively charged liposomes, incorporation of phosphatidylserine within the liposomal bilayer can promote higher delivery of liposomes and their entrapped moiety to local draining lymph nodes compared to other negatively charged liposomes. This enhanced trafficking of small PS liposomes may be the result of macrophage uptake with PS serving as a recognition signal for phagocytosis by macrophages [26] and triggering recognition by macrophages [27] with PS liposomes, thus potentially mimicking the effects of apoptotic cells on these phagocytes to induce the secretion of anti-inflammatory molecules and to inhibit the maturation of dendritic cells [28].

PS is recognised to exhibit anti-inflammatory properties. During apoptosis, exposure of PS on the outer leaflet of the cytoplasmic membrane acts as an apoptotic signal for phagocytes. Phosphatidylserine recognition of activated phagocytes inhibits pro-inflammatory cytokine

production such as Interleukin 6 (IL6) and Prostaglandin E2 (PGE2), in Interleukin 1 beta (IL-1β) stimulated *in vitro* and *in vivo* animal models [29], through induction of active anti-inflammatory or suppressive properties in macrophages [30]. Indeed, a previous study has shown the effects of PEGylated PS-containing liposomes on the expression of two inflammation associated cytokines, tumour necrosis factor-α (TNF-α) and transforming growth factor-β (TGF-β), in the murine macrophage-like cell line RAW 264.7 [31]. These results demonstrated that the incorporation of PEGylated PS-containing liposomes into cells decreased compared with non-PEGylated PS liposomes, suggesting that the interactions between PS and the PS receptors that influence TNF-α and TGF-β levels were differentially affected by the PEGylation of PS liposomes [31, 32]. As PEGylated PS containing liposomes are capable of efficiently inhibiting the inflammatory cytokine TNF-α without upregulating TGF-β mRNA [33], they demonstrate promising anti-inflammatory effect. Within our studies, we also demonstrate reduced uptake when we PEGylate the PS containing liposomes.

Our results also investigate the potential of the avidin/biotin complexation to enhance retention at the lymphatics. Several published studies previously have shown the ability of the avidin/biotin system to effectively target to the lymph nodes [34, 35]. For example, biotin coated liposomes were injected subcutaneously in both hind feet of rabbits, followed by an adjacent injection of avidin; 14 % of injected biotinylated liposomes retained at popliteal lymph nodes and 2 % in iliac lymph node after 24 h. Animals without avidin injection showed only 2 % and 0.3 % injected liposomes retention in popliteal and iliac lymph node respectively [10]. Moreover, a study in ovarian cancer bearing rats showed accumulation and retention of liposomes in the mediastinal lymph nodes and diaphragm for up to 22 h post-injection i.p. of biotinylated liposomes followed by avidin. Whereas, without an adjacent avidin injection, the biotin-liposomes quickly cleared the peritoneal cavity of the tumor-bearing rat by moving through the lymph nodes and returning to the blood circulation [34]. As demonstrated in our studies, a major advantage of using the biotin-liposome/avidin system within our studies is the prolonged retention and uptake by secondary lymph nodes such as mesenteric and inguinal lymph node due to the formation of avidin/biotin complex *in vivo* at the lymph node level [10]. Upon trafficking of the biotinylated liposomes to these lymphatics, aggregation of the biotin-coated liposomes with avidin occurs. These aggregated liposomes then become trapped in this or the next encountered lymph node [10]. This mechanism resulted in enhanced delivery and retention to the lymphatics of both the liposomes and entrapped protein. This re-directing of the liposomes and retention of non-adjuvanted liposomes did not induce notable immune responses. For example, if these formulations were to be benchmarked to a known liposomal adjuvant prepared from dimethyldioctadecylammonium bromide (DDAB) and trehalose 6,6'-dibehenate (TDB) using the H56 antigen IFN-g responses in the spleen can be 15 times higher whilst IL-17 is 6 times high [36]. There

is the potential that the liposomes may have driven a different type of immune response (e.g. IL-4 and IL-10); however given the biotin is conjugated to the liposomes via PEG, it is possible that this, combined with the lack of immune-stimulatory lipids within the formulation, circumvents any adjuvant activity.

Furthermore, within this study we demonstrate the application of microfluidics to formulate lymphatic targeting liposomes incorporating protein. A known issue during liposomes manufacturing is the low loading and protein degradation linked to the harsh conditions during sonication as traditional manufacturing methods, high shear stress conditions and high temperatures [37]. Within this work, biotinylated liposomes were successfully manufactured using microfluidics giving various advantages over traditional lipid thin film hydration method followed by extrusion. Microfluidics has been shown to be quick with reproducible vesicle sizes obtained with protein loading being markedly higher (up to 10 fold depending on the liposome formulation) in comparison with traditional passive loading technique [38].

Overall, a biotin/avidin complex mechanism can promote lymphatic targeting and prolong the retention of liposomes at lymph nodes after intramuscular injection, offer opportunities to deliver drugs or proteins to enhance lymphatic drug targeting. This targeting strategy does not translate to enhanced immune responses after vaccination with the DSPC:Chol:PS-biotin/avidin formulation.

Conclusions

Overall, our biodistribution studies confirm that the liposome formulations drained from the site of injection at different rates according to the composition, charge and size. However, upon clearing the injection site, the choice of lipid within the formulation also had an impact as liposomes containing PS showed higher accumulation at the draining lymph node compared to other anionic lipids. This may be due to preferential phagocytosis of the PS containing liposomes. Saturation of lymphatic uptake and lymph node localisation did not occur over a large liposomal lipid dose range (up to 480 μg/dose). These studies demonstrate that employing PS within small liposomes can promote lymphatic targeting after intramuscular injection. These liposomes were then surface modified with biotin so that avidin-biotin complexes were formed at the draining lymphatics to promote retain of these liposomes. Using this avidin/biotin liposome system and employing microfluidics to manufacture liposomal formulations lead to production of smaller sized liposomes that can deliver loaded model protein to lymph node effectively.

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Supporting information Available

Data presented in this publication can be found at [DOI to be provided on acceptance of manuscript].

References

1. Riquet, M., et al., *[Anatomy, micro-anatomy and physiology of the lymphatics of the lungs and chest wall].* Rev Pneumol Clin, 2013. **69**(2): p. 102-10.
2. McCance, K.L. and S.E. Huether, *Pathophysiology : the biologic basis for disease in adults and children*. 6th ed. 2010, Maryland Heights, Mo.: Mosby Elsevier. xxvi, 1838 p.
3. Jiang, H., Q. Wang, and X. Sun, *Lymph node targeting strategies to improve vaccination efficacy.* Journal of Controlled Release, 2017. **267**: p. 47-56.
4. Randolph, G.J., V. Angeli, and M.A. Swartz, *Dendritic-cell trafficking to lymph nodes through lymphatic vessels.* Nature Reviews Immunology, 2005. **5**: p. 617.
5. Liu, H., et al., *Structure-based programming of lymph-node targeting in molecular vaccines.* Nature, 2014. **507**: p. 519.
6. Kim, S.-Y., et al., *Synthetic vaccine nanoparticles target to lymph node triggering enhanced innate and adaptive antitumor immunity.* Biomaterials, 2017. **130**: p. 56-66.
7. Moghimi, S.M., et al., *Surface engineered nanospheres with enhanced drainage into lymphatics and uptake by macrophages of the regional lymph nodes.* 1994. **344**(1): p. 25-30.
8. Slütter, B., et al., *Adjuvant effect of cationic liposomes and CpG depends on administration route.* 2011. **154**(2): p. 123-130.
9. Oussoren, C., et al., *Lymphatic uptake and biodistribution of liposomes after subcutaneous injection.: II. Influence of liposomal size, lipid composition and lipid dose.* 1997. **1328**(2): p. 261-272.
10. Phillips, W.T., R. Klipper, and B. Goins, *Novel method of greatly enhanced delivery of liposomes to lymph nodes.* J Pharmacol Exp Ther, 2000. **295**(1): p. 309-13.
11. Phillips, W.T., R. Klipper, and B. Goins, *Novel method of greatly enhanced delivery of liposomes to lymph nodes.* Journal of Pharmacology and Experimental Therapeutics, 2000. **295**(1): p. 309-313.
12. Bangham, A.D., M.M. Standish, and J.C. Watkins, *Diffusion of univalent ions across the lamellae of swollen phospholipids.* J Mol Biol, 1965. **13**(1): p. 238-52.
13. Torr, E.E., et al., *Apoptotic cell-derived ICAM-3 promotes both macrophage chemoattraction to and tethering of apoptotic cells.* Cell Death Differ, 2012. **19**(4): p. 671-9.
14. Kaur, R., et al., *Effect of incorporating cholesterol into DDA:TDB liposomal adjuvants on bilayer properties, biodistribution, and immune responses.* Mol Pharm, 2014. **11**(1): p. 197-207.
15. Henriksen-Lacey, M., et al., *Liposomal cationic charge and antigen adsorption are important properties for the efficient deposition of antigen at the injection site and ability of the vaccine to induce a CMI response.* 2010. **145**(2): p. 102-108.
16. Henriksen-Lacey, M., et al., *Liposomes based on dimethyldioctadecylammonium promote a depot effect and enhance immunogenicity of soluble antigen.* 2010. **142**(2): p. 180-186.
17. Fadok, V.A., et al., *The role of phosphatidylserine in recognition of apoptotic cells by phagocytes.* Cell Death Differ, 1998. **5**(7): p. 551-62.
18. Dixon, J.B.J.T.i.E. and Metabolism, *Lymphatic lipid transport: sewer or subway?* 2010. **21**(8): p. 480-487.
19. Breslin, J.W.J.M.r., *Mechanical forces and lymphatic transport.* 2014. **96**: p. 46-54.

20. Henriksen-Lacey, M., A. Devitt, and Y.J.J.o.c.r. Perrie, *The vesicle size of DDA: TDB liposomal adjuvants plays a role in the cell-mediated immune response but has no significant effect on antibody production.* 2011. **154**(2): p. 131-137.
21. Brewer, J.M., et al., *Vesicle size influences the trafficking, processing, and presentation of antigens in lipid vesicles.* 2004. **173**(10): p. 6143-6150.
22. Forbes, N., et al., *Rapid and scale-independent microfluidic manufacture of liposomes entrapping protein incorporating in-line purification and at-line size monitoring.* 2019. **556**: p. 68-81.
23. Hawley, A., S. Davis, and L. Illum, *Targeting of colloids to lymph nodes: influence of lymphatic physiology and colloidal characteristics.* Advanced Drug Delivery Reviews, 1995. **17**(1): p. 129-148.
24. Porter, C. and W. Charman, *Transport and absorption of drugs via the lymphatic system.* Advanced drug delivery reviews, 2001. **50**(1-2): p. 1-2.
25. Oussoren, C., et al., *Lymphatic uptake and biodistribution of liposomes after subcutaneous injection.: II. Influence of liposomal size, lipid composition and lipid dose.* Biochimica et Biophysica Acta (BBA)-Biomembranes, 1997. **1328**(2): p. 261-272.
26. Fadok, V.A., et al., *The role of phosphatidylserine in recognition of apoptotic cells by phagocytes.* Cell death and differentiation, 1998. **5**(7): p. 551-562.
27. Oussoren, C., et al., *Lymphatic uptake and biodistribution of liposomes after subcutaneous injection. II. Influence of liposomal size, lipid compostion and lipid dose.* Biochim Biophys Acta, 1997. **1328**(2): p. 261-72.
28. Otsuka, M., S. Tsuchiya, and Y. Aramaki, *Involvement of ERK, a MAP kinase, in the production of TGF-beta by macrophages treated with liposomes composed of phosphatidylserine.* Biochem Biophys Res Commun, 2004. **324**(4): p. 1400-5.
29. Yeom, M., et al., *Phosphatidylserine inhibits inflammatory responses in interleukin-1β–stimulated fibroblast-like synoviocytes and alleviates carrageenan-induced arthritis in rat.* Nutrition research, 2013. **33**(3): p. 242-250.
30. Fadok, V.A., et al., *Macrophages that have ingested apoptotic cells in vitro inhibit proinflammatory cytokine production through autocrine/paracrine mechanisms involving TGF-beta, PGE2, and PAF.* The Journal of clinical investigation, 1998. **101**(4): p. 890-898.
31. Quan, H., et al., *Modulation of the anti-inflammatory effects of phosphatidylserine-containing liposomes by PEGylation.* Journal of Biomedical Materials Research Part A, 2017. **105**(5): p. 1479-1486.
32. Krahling, S., et al., *Exposure of phosphatidylserine is a general feature in the phagocytosis of apoptotic lymphocytes by macrophages.* Cell death and differentiation, 1999. **6**(2): p. 183.
33. Xu, Z., G. Smejkal, and R. Marchant. *Interaction of plasma proteins with liposomes of different lipid composition*. in *ANNUAL MEETING-SOCIETY FOR BIOMATERIALS IN CONJUNCTION WITH THE INTERNATIONAL BIOMATERIALS SYMPOSIUM*. 1998. SOCIETY FOR BIOMATERIALS.
34. Zavaleta, C.L., et al., *Use of avidin/biotin-liposome system for enhanced peritoneal drug delivery in an ovarian cancer model.* International Journal of Pharmaceutics, 2007. **337**(1): p. 316-328.
35. Medina, L.A., et al., *Avidin/biotin-liposome system injected in the pleural space for drug delivery to mediastinal lymph nodes.* Journal of Pharmaceutical Sciences, 2004. **93**(10): p. 2595-2608.
36. Wilkinson, A., et al., *Lipid conjugation of TLR7 agonist Resiquimod ensures co-delivery with the liposomal Cationic Adjuvant Formulation 01 (CAF01) but does not enhance immunopotentiation compared to non-conjugated Resiquimod+CAF01.* Journal of Controlled Release, 2018. **291**: p. 1-10.
37. Tamber, H., et al., *Formulation aspects of biodegradable polymeric microspheres for antigen delivery.* Advanced drug delivery reviews, 2005. **57**(3): p. 357-376.

38. Cullis, P., et al., *Generating and loading of liposomal systems for drug-delivery applications.* Advanced drug delivery reviews, 1989. **3**(3): p. 267-282.