Liposome-based vaccines for immune modulation: from antigen selection to nanoparticle design

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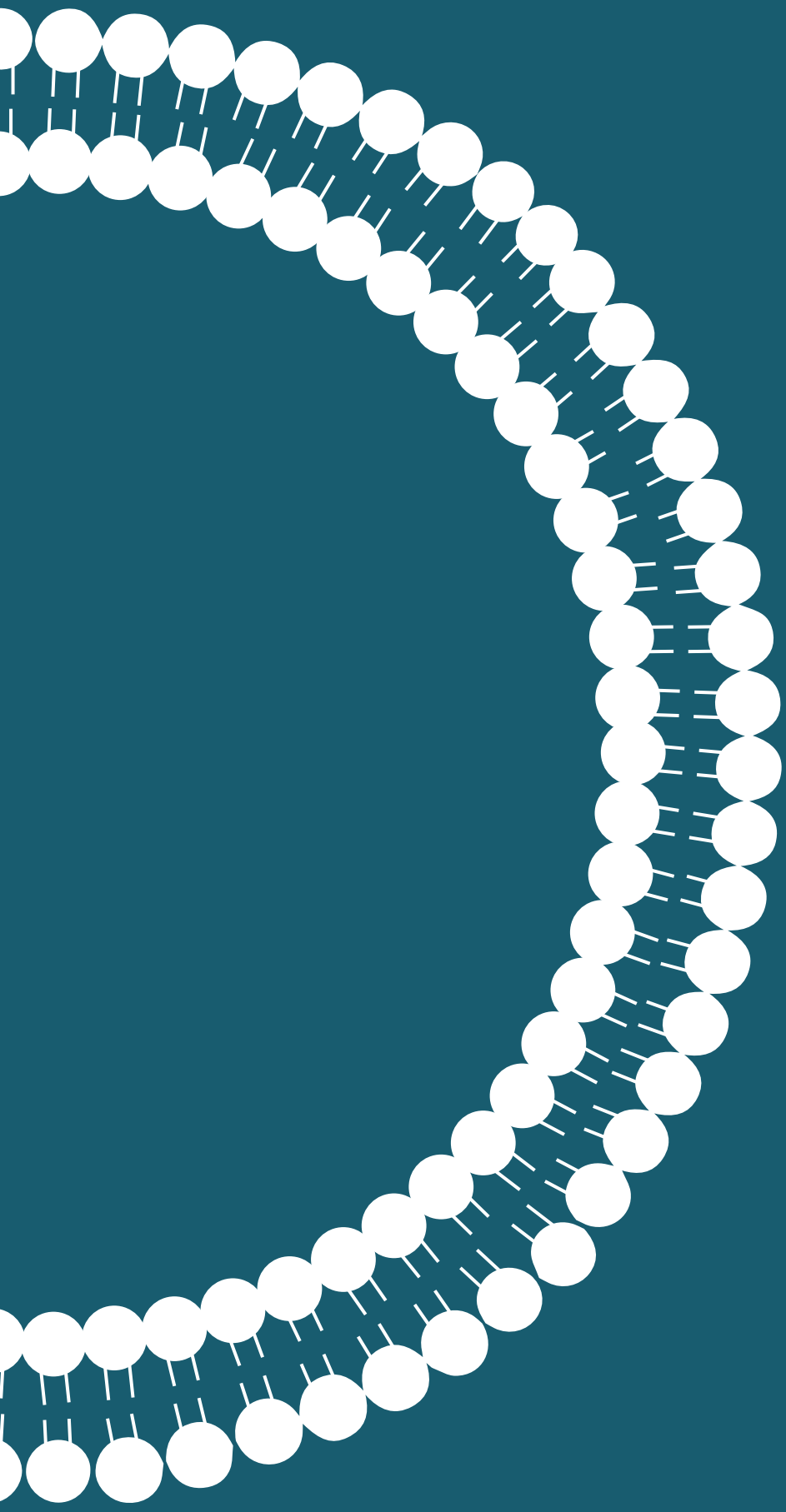

Chapter 5

The Use of a Staggered Herringbone Micromixer for the Preparation of Rigid Liposomal Formulations Allows Efficient Encapsulation of Antigen and Adjuvant

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ABSTRACT

Anionic liposomal formulations have previously shown to have intrinsic tolerogenic capacity and these properties have been related to the rigidity of the particles. The combination of highly rigid anionic liposomes to deliver tolerogenic adjuvants and antigen peptides has potential applications for the treatment of autoimmune and inflammatory diseases. However, the preparation of these highly rigid anionic liposomes using traditional methods such as lipid film hydration presents problems in terms of scalability and loading efficiency of some costly tolerogenic adjuvants like 1-α,25-dihydroxyvitaminD3. Here we propose the use of an off-the-shelf staggered herringbone micromixer for the preparation of these formulations and perform a systematic study on the effect of temperature and flow conditions on the size and polydispersity index of the formulations. Furthermore, we show that the system allows for the encapsulation of a wide variety of peptides and significantly higher loading efficiency of 1-α,25-dihydroxyvitaminD3 compared to the traditional lipid film hydration method, without compromising their non-inflammatory interaction with dendritic cells. Therefore, the microfluidics method presented here is a valuable tool for the preparation of highly rigid tolerogenic liposomes in a fast, size-tuneable, and scalable manner.

Keywords

liposomes, rigidity, herringbone micromixer, microfluidics, vitaminD3, tolerance

Abbreviations

DiD, DiIC18(5); 1,1′-dioctadecyl-3,3,3′,3′- tetramethylindodicarbocyanine, 4-chlorobenzenesulfonate; DLS, dynamic light scattering; DSPC, 1,2-distearoyl-*sn*-glycero-3-phosphocholine; DSPG, 1,2-Distearoyl-*sn*-glycero-3-phosphoglycerol; FRR, flow rate ratio; GRAVY, grand average of hydropathy; HPLC, high-performance liquid chromatography; LE%, loading efficiency; LFH, lipid film hydration; MWCO, molecular weight cut-out; PB, phosphate buffer; PdI, polydispersity index; TEM, transmission electron microscopy; TFA, trifluoroacetic acid; TFR, total flow rate; TIPS, triisopropylsilane; UPLC, ultra-high performance liquid chromatography; VD3, 1-α,25-dihydroxyvitaminD3

INTRODUCTION

Liposomes are nanometre-sized vesicles formed by a lipid bilayer enclosing an aqueous core and are used for various applications, including vaccination. Whereas cationic liposomes and lipid nanoparticle formulations have shown to have immune stimulatory properties[1] anionic liposomes might induce immune tolerance. We have previously shown that 1,2-Distearoyl-*sn*-glycero-3-phosphoglycerol (DSPG)-containing liposomes carrying peptide antigens are able to induce T regulatory cells, which are key mediators of peripheral tolerance, and was able to arrest development of atherosclerosis in mice[2]. The tolerogenic capacity of these DSPG formulations is, in part, due to the high rigidity of these liposomes as this impacts both the uptake of the formulation by professional antigen presenting cells, such as dendritic cells, and on the capacity of the formulation to induce tolerogenic immune responses[3].

Although the physicochemical properties of liposomal formulations can influence their capacity to induce a tolerogenic immune response, the generation of a strong tolerogenic response might require the inclusion of tolerogenic adjuvants. Immune modulatory molecules such as 1-α,25-dihydroxyvitaminD3 (VD3), rapamycin or retinoic acid have been widely studied for their capacity to induce tolerogenic dendritic cells and may therefore represent interesting adjuvants for tolerogenic vaccines[4-6]. However, despite their high hydrophobicity index, the loading efficiency of these molecules, specially VD3, into highly rigid anionic liposomes using the traditional lipid film hydration method is surprisingly low (Table 1) with only a small fraction (<10%) of the VD3 being loaded into the liposomes. Moreover, the traditional liposome preparation of lipid film hydration also has limitations regarding the scalability and batch to batch variability.

Table 1. Loading efficiency of tolerogenic adjuvants into anionic rigid liposomes using the lipid film hydration method. The table summarizes the average loading efficiency of at least 2 separate batches of liposomes (n=2)

	Loading efficiency % (± SD)
Rapamycin	7.1 (± 5.1)
Retinoic acid	38.6 (± 0.9)
1-α,25-dihydroxyvitaminD3	8.08 (± 10.1)

Due to the promising application of highly rigid anionic liposomes in the field of antigen-specific tolerance and the need for methods to efficiently load tolerogenic adjuvants, we developed a microfluidics-based approach.

Microfluidics allows the high-throughput and scalable manufacture of liposomal formulations. In these systems, the sudden change in solvent polarity in a

micrometre-sized channel triggers the nanoprecipitation of the phospholipids, forming lipid bilayers with more thermodynamically stable structures[7] and may encapsulate more antigen and adjuvant in the process. However, the available commercial microfluidics systems have limitations for the preparation of highly rigid liposomal formulations. These formulations need to be prepared above the transition temperature of the phospholipids, which in the case of liposomes containing 1,2-distearoyl-*sn*-glycero-3-phosphocholine (DSPC) and DSPG is 55˚C. The design of the microfluidics systems makes difficult to accurately define the temperature in the microchannel. Here we described a method with an off-the-shelf glass staggered herringbone micromixer that allows the preparation of highly rigid anionic liposomes loaded with VD3. This system offers significant advantages over the lipid film hydration method including a markedly improved loading efficiency of VD3.

MATERIALS AND METHODS

Materials

Staggered herringbone micromixer was purchased from Darwin Microfluidics (Paris, France) cat# LTF-012.00-4264. Both the aqueous and organic phases were loaded into Hamilton glass syringes obtained from Sigma-Aldrich (Zwijndrecht, The Netherlands) and the inlet flow was controlled by two single channel syringe pumps (ProSense, Oosterhout, The Netherlands). 1,2-distearoyl-*sn*-glycero-3-phosphocholine (DSPC) and 1,2-distearoyl-*sn*-glycero-3-phospho-(1'-rac-glycerol) (DSPG) were purchased from Avanti Polar Lipids (Alabaster, USA). Cholesterol was obtained from Sigma-Aldrich (Zwijndrecht, The Netherlands). 1-α,25-dihydroxyvitaminD3 was purchased from Sigma-Aldrich (Zwijndrecht, The Netherlands). Peptides were synthesized in house by solid-phase peptide synthesis (SPPS) using the CEM microwave-assisted automated peptide synthesizer Liberty Blue. Ethanol absolute was purchased from Biosolve (Valkenswaard, The Netherlands). Float-A-lyzers 100,000Da MWCO dialysis tubes were purchased from Sigma-Aldrich (Zwijndrecht, The Netherlands). Whatman Nucleopore polycarbonate track-etched membranes were obtained from Sigma-Aldrich (Zwijndrecht, The Netherlands).

Methods

Preparation of highly rigid anionic liposomes using staggered herringbone micromixer

The organic phase for the preparation of anionic liposomal formations consisted on DSPC:DSPG:Cholesterol dissolved in ethanol absolute in a molar ratio 4:1:2.

The lipid concentration on the organic phase was 10 mg/mL unless specified otherwise. The aqueous phase consisted of Phosphate Buffer (PB) 10 mM pH 7.4, in the case of peptide-loaded liposomes, the peptides were included in the aqueous phase at a concentration of 100 μg/mL. In the case of liposomes loaded with 1α,25-dihydroxyvitaminD3 or labelled with the lipophilic dye DiD, the adjuvant or fluorescent label were included in the organic phase together with the lipids. The concentration of 1α,25-dihydroxyvitaminD3 in the organic phase was 150 μg/mL. The organic and aqueous phases were injected into the herringbone micromixer as schematically depicted in Figure 1. The flow rate ratio (FRR) between the aqueous and the organic phase was varied from 2:1 to 5:1 (Table S1) and the total flow rate (TFR), defined as the sum of the aqueous flow rate and the organic flow rate, was also varied from 100 μL/min to 900 μL/min in order to determine the effect of flow conditions on particle size and polydispersity index (PdI). All formulations were prepared at a temperature above the gel-liquid phase transition temperature of the phospholipids in the formulation unless mentioned otherwise. The temperature was set by submerging the micromixer in a water bath at 60˚C. The temperature of the water bath was controlled by using a temperature probe connected to a heating plate. Formulations were dialyzed against 400 mL of PB 10 mM pH 7.4 overnight with constant stirring using Float-A-lyzer dialysis kit (MWCO 100,000 Da) to remove traces of ethanol and non-encapsulated molecules.

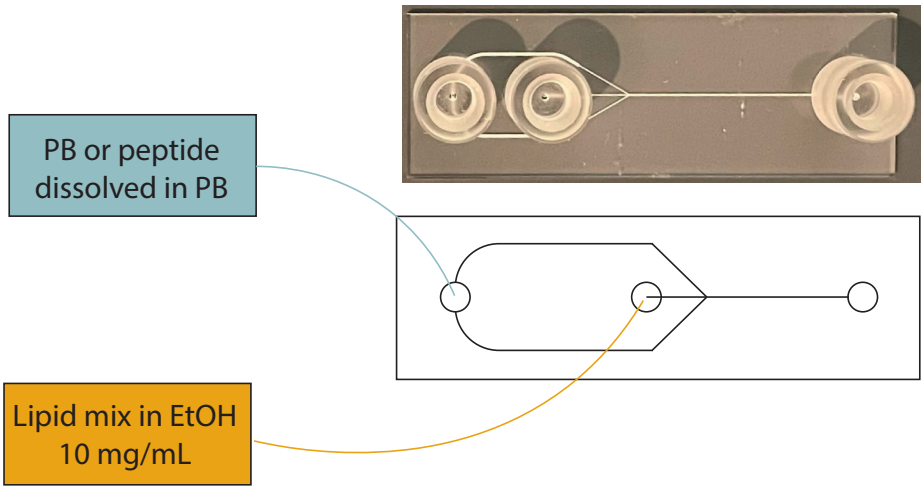

Figure 1. Microfluidics system setup. The staggered herringbone micromixer consists of a glass chip with two inlet ports, one for the organic phase and another for the aqueous phase. The organic and the aqueous phase are mixed in a microchannel of 200 μm width and 80 μm depth. The microchannel presents a series of chevrons (mixing element) with a short arm (1/3 channel width) and a long arm (2/3 of channel width). The height of each chevron is 30μm. The microchannel presents a total of 180 chevron elements and the length of the mixing channel is 28.7mm.

Preparation of liposomes using lipid film hydration method

For VD3-loaded liposomes, a total of 5 mg phospholipids and cholesterol dissolved in chloroform and 50 μg VD3 were mixed in a round bottom flask in a molar ratio of 4:1:2 (DSPC:DSPG:Cholesterol). In the case of DiD-labelled liposomes, a total of 6.6 mg of phospholipids and cholesterol at a molar ratio of 4:1:2 (DSPC:DSPG:Cholesterol) and 0.1 mol% of DiD dissolved in chloroform were mixed in a round bottom flask. A dry lipid film was formed by removing the chloroform in a rotary evaporator (180 mbar, 15 minutes) at 40˚C. The dry lipid film was hydrated with 1 mL PB 10 mM pH 7.4 in a rotary evaporator (atmospheric pressure, rotation only) at 60˚C for 30 minutes. The product of the lipid film hydration is a suspension of large multilamellar vesicles. Samples were extruded 4 times through 400 nm and 200 nm stacked track-etch polycarbonate membranes (Whatman® Nucleopore™, GE Healthcare, Little Chalfont, UK) at high pressure using an extruder (LIPEX Extruder, Northern Lipids Inc., Canada) connected to a water bath at 60˚C. The formulations were dialyzed overnight against 400 mL of PB 10 mM pH 7.4 using Flot-A-lyzer dialysis kit (MWCO 100,000 Da).

Liposome characterization – DLS

Dynamic Light Scattering (DLS) was performed using a Zetasizer NanoZS (Malvern Panalytical, UK) to measure the z-average hydrodynamic diameter and PdI of the formulations. The same instrument was used to determine the ζ-potential by means of laser doppler electrophoresis. When the measurement was performed prior to dialysis, the method was adjusted to consider the changes in the viscosity and refractive index of the dispersant due to the presence of ethanol in the samples (Table S2).

Liposome characterization – UPLC

Reverse phase ultra-high performance liquid chromatography (UPLC) was used for the quantification of the encapsulated peptide, VD3 and total lipids. For quantification of the lipophilic compound VD3, a sample of the formulation was dried under a N_2 stream, resuspended in the same volume of ethanol, and subsequently injected into a 1.7 μm BEH C18 column (2.1 x 50 mm, Water ACQUITY UPLC, Waters, MA, USA). For the measurement, 10uL of sample was injected into the UPLC column. Column temperature was set at 40˚C. The mobile phases consisted of milliQ water with 0.1% TFA (solvent A) and acetonitrile with 0.1% TFA (solvent B). After sample injection, a linear gradient of solvent B from 5% to 95% was applied to the column for 7 minutes at a flow rate of 0.5 mL/min, followed by 95% solvent B for 2 minutes and 5% solvent B 95% solvent A for 3 minutes. Peptides were detected by absorbance at 220 nm, VD3 was detected by absorbance at 252 nm using ACQUITY UPLC TUV detector and lipids were detected using ACQUITY UPLC Evaporative Light

Scattering detector (ELSD). Loading efficiency was calculated as the total amount of peptide or VD3 after dialysis divided by the total amount of peptide before dialysis (for formulations prepared with microfluidics) or before extrusion (for formulations prepared with lipid film hydration method).

Liposome characterization – Negative staining Transmission Electron Microscopy (TEM)

For the characterization of liposomes loaded with VD3 by Transmission Electron Microscopy (TEM), a sample of liposomal formulation was deposited on carbon-Formvar coated 200 mesh copper grids (Electron Microscopy Sciences, USA) for 30 seconds and subsequentially stained with a contrast solution of 1% uranyl acetate for 30 seconds. The coated grids were left to dry overnight at room temperature before imaging. Images were taken in a JEM1400 plus Transmission Electron Microscope operating at 80 kV and fitted with a CCD camera.

Peptide synthesis

Peptides were synthesized in-house by solid-phase peptide synthesis (SPPS) using a Liberty Blue microwave-assisted automated peptide synthesizer. Synthesis scale was 0.1 mmol using an S-RAM Tentagel resin. Fmoc-deprotection was done with dimethylformamide (DMF) containing 20% piperidine. After synthesis peptides were acetylated using pyridine and acetic anhydride (1:1 v/v). Peptide cleavage from the resin was performed by incubating the resin for 1h with a mixture of 95% (v/v) trifluoroacetic acid (TFA), 2.5% (v/v) triisopropylsilane (TIPS) and 2.5% (v/v) water. Peptides were precipitated using ice-cold diethyl ether followed by centrifugation. Peptides were subsequently purified by reverse-phase high performance liquid chromatography (HPLC) using a Kinetic Evo C18 column. Purity was assessed by liquid chromatography-mass spectrometry (LC-MS).

Net charge of peptides at pH 7.4 was calculated using Innovagen peptide property calculator. The grand average of hydropathy (GRAVY) of peptides was calculated as the sum of hydropathy values of the amino acids divided by the peptide length. The sequences and physicochemical properties of the peptides can be found in Table S3.

Human monocyte-derived dendritic cell culture and activation

Peripheral blood monocytes were isolated from buffy coats or fresh blood and differentiated into monocyte-derived dendritic cells (moDCs) as previously described[8]. To assess moDC liposome uptake 50-200 x 10^3 immature DCs were incubated with DiD-labelled formulations prepared by either the lipid-film hydration method or the staggered herringbone micromixer at a lipid concentration of 10 μg/mL or 30 μg/mL for 4 hours in IMDM (Thermo Fisher Scientific) 5% FCS (Sigma-

Aldrich, St Louis, Missouri). Cells were washed and liposome uptake was measured by flow cytometry on a FACS Canto. Uptake was quantified using percentages of DiD-positive moDCs. For determination of moDC maturation, immature DCs were cultured in IMDM (Thermo Fisher Scientific) 5% FCS (Sigma-Aldrich, St Louis, Missouri) supplemented with 500 U/mL granular-macrophage colony stimulating factor (GM-CSF) only, or with 100 ng/mL lipopolysaccharide (LPS) derived from *E. coli* strain O111-B4 (Sigma-Aldrich) and GM-CSF, in the presence or absence of 10 or 30 µg/mL liposomes for 24 hours. Stimuli were washed away and DCs were stained with a cocktail of the following markers: anti-CD83-phycoerythrin (PE) (BD Biosciences, Franklin Lake, New Jersey), anti-CD86-brilliant violet 421 (BV421) (BD), anti-HLA-DR-peridinin-chlorophyll-protein-Cy5.5 (PerCP-Cy5.5) (BD), and anti-CD14-PE-Cy7 (Biolegend, San Diego, California). Activation of moDCs was determined by flow cytometric analysis on a FACS Fortessa (BD). For compensation, single marker-fluorochrome stainings were included. Flow cytometry data was analysed using FlowJo software (Treestar, Ashland, Oregon).

Statistics

Statistical differences between groups were analysed by either unpaired T-test or one-way ANOVA followed by Tukey´s multiple comparisons test. Statistical correlation was tested using Pearson's correlation test. P-values below 0.05 were considered significant. All analysis were performed using GraphPad Prism 8.1.1 for Windows (GraphPad Software, San Diego, California, USA).

RESULTS

Temperature of the system is a key parameter for the preparation of monodisperse highly rigid anionic liposomes by microfluidics

One of the challenges of the preparation of highly rigid liposomes is the relatively high transition temperature of the phospholipids in these formulations. Therefore, during the preparation of these liposomes using microfluidics, the aqueous and organic phases need to be above this temperature. A drawback of most microfluidic systems is the inability to properly control the temperature, complicating the controlled nanoprecipitation of lipids with a high transition temperature. Indeed, DSPC:DSPG:Cholesterol liposome prepared at room temperature resulted in relatively large liposomes (Figure 2A) and high PdI (Figure 2B). Merely heating the inlet solutions before injection in the micromixer did not correct this issue (Figure 2A, B), however submerging the micromixer in water bath at 60˚C resulted in monodisperse liposomes with an average size of 120 nm and a PdI below 0.2

(Figure 2A, B). This shows that controlling the temperature of the entire system allows the production highly rigid liposomes with great reproducibility.

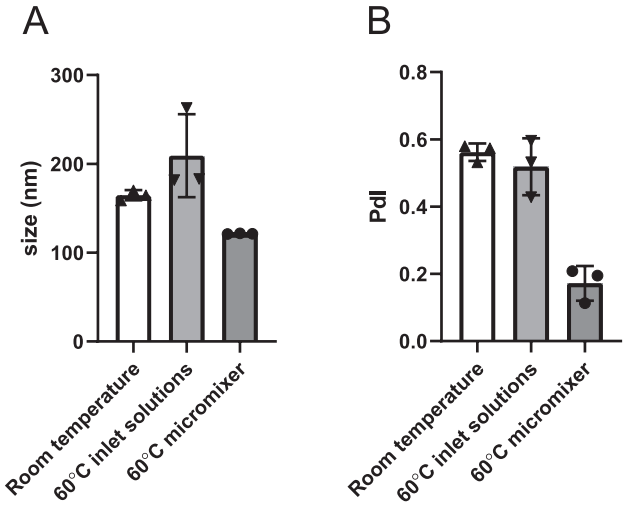

Figure 2. Importance of temperature control in micromixer. Effect of temperature on (A) size and (B) PdI of DSPC:DSPG:Cholesterol liposomes prepared in staggered herringbone micromixer. Formulations were either prepared at room temperature, by heating the inlet solutions at 60°C before injections or by submerging the micromixer in a temperature-controlled water bath at 60°C. All formulations were prepared at a TFR of 500 μL/min and a FRR of 2:1. Graphs show three replicate measurements from the same batch of formulation.

The flow rate ratio and not the total flow rate determine the particle size during preparation

Having established that the microfluidics system allows for the formation of monodisperse rigid liposomes, we next addressed whether this is scalable and tuneable. To increase the output, we varied the total flow rate (TFR) of the system from 100 μl/min to 900 μl/min. Remarkably, the average size of the liposomes and PdI were not significantly influenced by the TFR (Figure 3A and 3B). This suggest that our system is robust even at higher flow rates, which may increase the scale-up potential of the system since higher volumes of formulations can be prepared in a short period of time.

Next, we adjusted the flow rate ratio (FRR) of water and organic phase while keeping the TFR constant at 500 μl/min. We observed that by increasing the flow rate in favour of the aqueous phase, the resulting liposomes exhibited a smaller average hydrodynamic diameter (Figure 3C) while remaining monodisperse (Figure

3D). Thus, the average size of liposomes is tuneable with our system by adjusting the FRR.

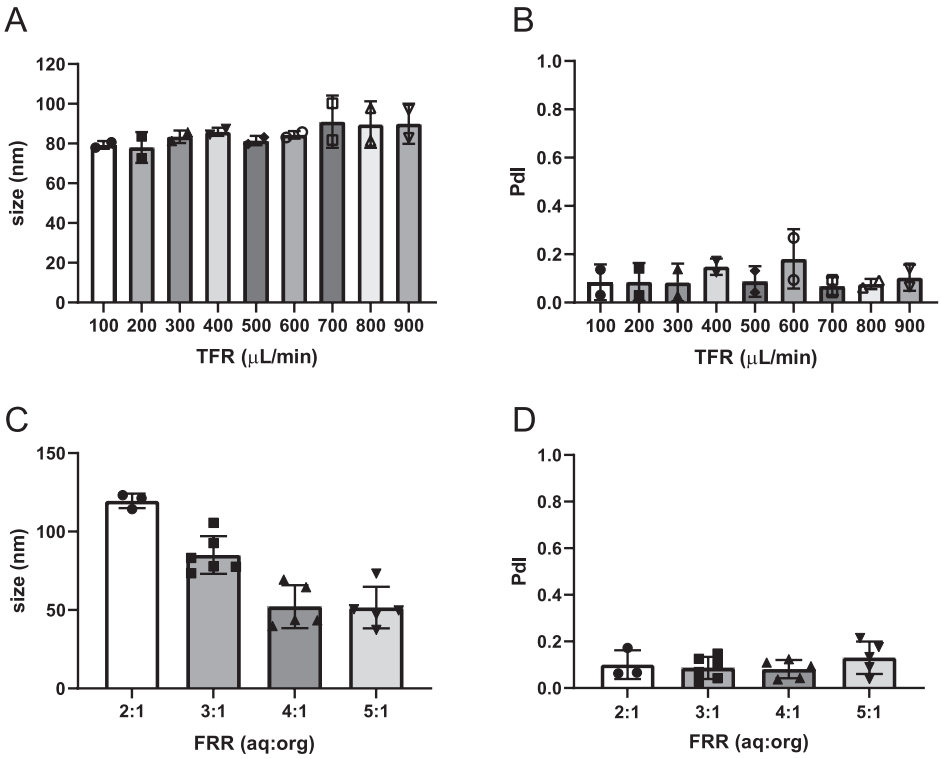

Figure 3. Effect of flow conditions on liposome characteristics. Effect of TFR on (A) average particle size and (B) PdI of DSPC:DSPG:Cholesterol formulations. To study the effect of TFR, formulations were prepared at a fixed FRR of 3:1. Effect of FRR on (C) average particle size and (D) PdI of DSPC:DSPG:Cholesterol formulations. To study the effect of the FRR, formulations were prepared using a fixed TFR of 500 μl/min. Each data point represents a separate batch of formulation. Graphs show data from independent batches of formulation and the mean of that data.

Loading efficiency of peptide antigen across a wide spectrum of charge and hydrophobicity

Next, we addressed whether the microfluidics system allows for incorporation of peptide antigens and adjuvants. Encapsulation of peptides can be particularly challenging as they can have a wide range of physicochemical properties. To address this, we selected 7 peptides (Table S3) with a wide range of net charge and hydrophobicity (expressed as the grand average of hydropathicity index, GRAVY) and determined the loading efficiency. We observed a wide variability in loading efficiency (Figure 4) and no statistical correlation between the loading

efficiency and the net charge (r = 0.65, p-value = 0.11) or GRAVY (r = 0.44, p-value = 0.32), however none of the peptides show a lower encapsulation than 10%.

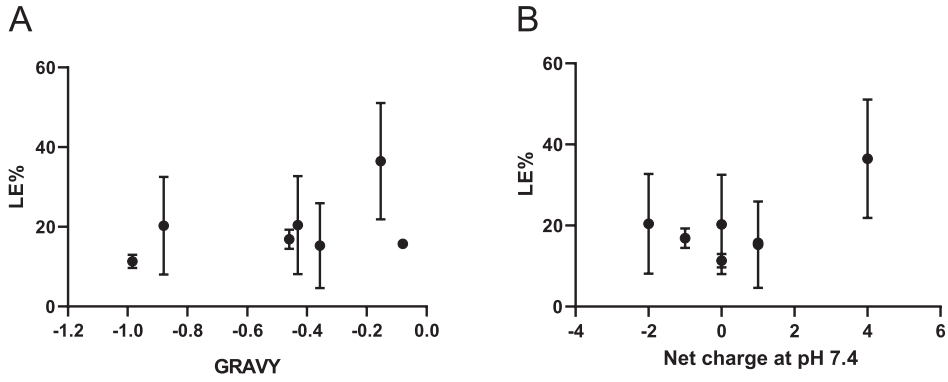

Figure 4. Effect of GRAVY and net charge of peptides in the loading efficiency (LE). (A) Loading efficiency of different peptides into DSPC:DSPG:Cholesterol liposomes vs GRAVY. (B) Loading efficiency vs net charge of peptides. The flow conditions for these formulations were set at 400 μL/min and a FRR of 2:1. Each data point represents the mean loading efficiency (± SD) of two independent batches of formulation.

The loading efficiency of VD3 is significantly improved when the highly rigid liposomes are prepared using the herringbone micromixer

A major drawback of the lipid film hydration method is the poor encapsulation of tolerogenic adjuvants into liposomes with a high phase transition temperature (Table 1). Therefore, we next addressed if the encapsulation efficiency of VD3 can be improved by using the staggered herringbone micromixer. Addition of VD3 to production process increased the size of the liposomes (Figure 5A) and the PdI (Figure 5B) compared to unloaded liposomes (Figure 2A), however size was comparable to the lipid film hydration equivalent (Figure 5A). Formulations prepared using the microfluidics system showed an increase in PdI and a slight decrease in the ζ-potential (Figure 5B and 5C) compared to the formulations prepared with lipid film hydration method. The microfluidics method, however, was vastly superior to the lipid film hydration method in the loading efficiency of VD3 (Figure 5C, 60% vs 2% $p < 0.0001$). Therefore, the changes in PdI and ζ-potential could be explained by the incorporation of VD3 into the liposomes. Importantly, TEM imaging suggests the high encapsulation efficiency is not a result of formation of VD3 micelles or aggregates (Figure 5E).

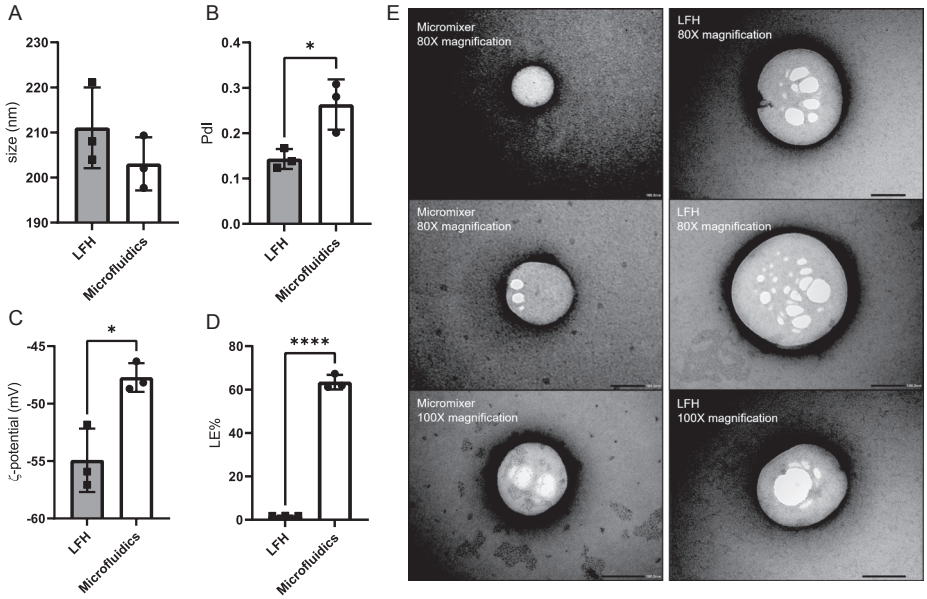

Figure 5. Comparison of VD3 incorporation into liposomes using lipid film hydration method vs microfluidics. (A) Average particle size, (B) PdI and (C) ζ-potential of DSPC:DSPG:Cholesterol liposome loaded with VD3 prepared using either the staggered herringbone micromixer or the gold-standard lipid-film hydration method (LFH) (n = 3). (D) Loading efficiency of VD3 for both methods. The flow conditions in the microfluidics system were set at TFR of 500 μL/min and a FRR of 3:1 (Aqueous:Organic). The final lipid and VD3 concentration in the formulations was 5 mg/ml and 50 μg/mL respectively for both the microfluidics and LFH formulations. (E) Transmission Electron Microscopy (TEM) images of VD3-loaded liposomes prepared with either the staggered herringbone micromixer or the lipid film hydration method. Black scale bars represent 100 nm. Graphs show mean, each data point represents an independent batch of formulation *p<0.05, ****p<0.0001 determined by unpaired t-test.

Exposure of monocyte-derived dendritic cells to the highly rigid anionic liposomes prepared with the micromixer does not induce DCs activation

We have previously shown that rigid anionic liposomes are more easily taken up by antigen presenting cells than fluid liposomes and have a propensity to induce tolerance[3]. In order for liposomes to affect the immune response, the uptake of the nanoparticles by dendritic cells is essential. We used monocyte-derived dendritic cells (moDCs) to assess if liposomes prepared using the traditional lipid film hydration method and the staggered herringbone micromixer are comparable in terms of cell uptake and biological effect. To assess cell uptake, we exposed moDCs to liposomes prepared with either lipid-film hydration method or the

staggered herringbone micromixer at two different lipid concentrations (10 and 30 μg/mL) for 4h. Both lipid film hydration prepared as well as microfluidics prepared liposomes were readily taken up by moDC. Albeit we observed a reduction in cell uptake for one of the batches prepared with microfluidics compared to the lipid film hydration method after 4h of incubation, we did not observe significant differences in uptake when a second batch of liposomes was tested (Figure 6A). Furthermore, we studied the capacity of the formulations to induce activation of moDCs. For this, cells were exposed to the formulations for 24h and CD86 and CD83 expression was assessed by flow cytometry. Results showed no significant differences in the activation makers studied (Figure 7C and 7D), indicating that these formulations prepared with either the lipid film hydration method or microfluidics, do not have immune-stimulatory or pro-inflammatory properties.

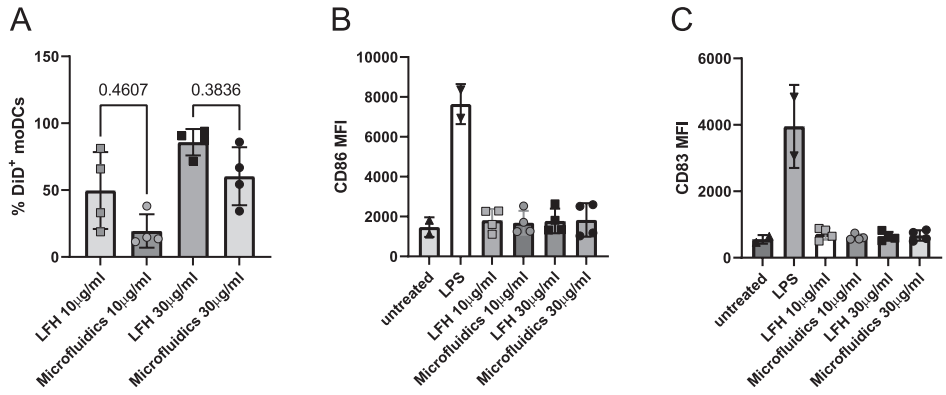

Figure 6. Effect of preparation method on uptake and cell activation. (A) Uptake by monocyte derived DC of DiD-labelled anionic liposomes (DSPC:DSPG:Cholesterol) prepared using lipid-film hydration method (LFH) or microfluidics after 4h and (B, C) resulting DC maturation after 24h of incubation. Panels B and C show the mean fluorescence intensity (MFI) of the activation markers (B) CD86 and (C) CD83. Data represent 2 moDC donors treated with two independently prepared batches at two different lipid concentrations (10 and 30 μg/mL). P-value determined by one-way ANOVA followed by Tukey´s multiple comparisons test.

DISCUSSION

Physicochemical parameters of liposomal formulations such as the rigidity can affect their ability to induce certain immune responses. In previous studies we have shown that highly rigid anionic liposomal formulations containing distearoyl-sn-glycero-3-phosphoglycerol (DSPG) are more efficient at delivering their cargo and inducing T regulatory cells[3]. The inclusion of tolerogenic adjuvants, such as VD3, in these formulations may potentiate the tolerogenic capacity of the liposomes. However, the loading of these molecules into highly rigid liposomes has proven to

be challenging using traditional liposome preparation methods. These liposome production methods also present other drawbacks, for example, formulations need to be prepared in batches, which is time consuming and leads to variability between batches[9,10]. On the other hand, they require sizing-down the multilamellar vesicles formed after the hydration of the dry lipid film in order to generate monodisperse unilamellar liposomal formulations. Sizing methods commonly used, such as extrusion, are not suitable for large-scale production due to the amount of energy required to pass litres of formulations through nm-size filters[11]. Furthermore, liposomes composed of phospholipids with high transition temperature also require temperature control during extrusion, which would increase the energy consumption of the manufacture process. Extrusion can also lead to the loss of costly material such as lipids, antigen and/or adjuvants. Microfluidics methods for the preparation of liposomes have the ability to overcome some of these limitations. In general, the formation of liposomes in these systems occurs when phospholipids dissolved in a water-miscible organic solvent mixes with an aqueous solution in a microchannel. The controlled mix of the organic and aqueous solvents triggers the nanoprecipitation of phospholipids that will first form lipid bilayer discs and then vesicles[7]. Highly rigid liposomes are composed of phospholipids with high transition temperature (55˚C or higher) and the solubility of these phospholipids in organic solvents is often poor at room temperature. Therefore, the control of temperature is a key parameter during liposome manufacture process both for the lipid film hydration method and microfluidics-based methods. Temperature control in commercial microfluidics systems often consist of heating the aqueous and organic solutions in the inlet syringes. However, this might not ensure that the temperature in the microchannel is the required working temperature and can lead to formulations[12] with high polydispersity or high inter-day variability due to changes in ambient temperature. The material and relatively small dimensions of the staggered herringbone micromixer used here allowed the tight control of the temperature during mixing by submerging the glass chip in a water bath at the desired temperature. We observed that a proper control of the mixing temperature is necessary in order to obtain monodisperse liposomal formulations and that these conditions cannot be achieved by heating the inlet solutions prior to injection.

Apart from liposomal rigidity, other parameters such as average particle size also have an impact on the biological activity of nanoparticle formulations. Liposome particle size can affect the biodistribution, cell uptake and immune responses[13]. For instance, studies have shown that particles with a size of 50 nm can induce different types of immune responses compared to those with an average particle size of 120 nm[14]. Therefore, the ability to prepare liposomal formulations with a controllable particle size is essential for any liposome preparation method.

We showed that the particle size of the liposomes prepared using the microfluidics method proposed here can be altered by changing the flow conditions, specifically the FRR between the aqueous solvent and the organic solvent. By increasing the proportion of the aqueous solvent, the average particle size of the formulations can be reduced without significantly affecting the PdI. The other flow parameter under study, the TFR, did not significantly affect the average particle size or PdI of the formulation, which highlights the scale up potential of this preparation method since up to 900 μL of formulation can be prepared per minute.

The ability to efficiently load cargo into liposomal formulations is also essential in a liposome preparation method. Loading peptide antigens into highly rigid liposomal formulations could allow the induction of an antigen-specific tolerogenic response since these formulations have previously shown to be more efficient at delivering their cargo[3]. We studied the loading efficiency of peptides with different net charges at pH 7.4 and different levels of hydrophilicity. Although no correlation was observed between the loading efficiency of the peptides and their net charge or GRAVY value, it is worth noting that the peptide with the higher positive charge (+4) also showed the highest loading efficiency, possibly related to the favourable electrostatic interaction between the peptide and the anionic liposomes. In any case, the loading efficiency above 10% observed for all the peptides is in line with what we have previously observed using the lipid film hydration method[2]. Contrary to what could be expected, the loading of lipophilic molecules with tolerogenic properties such as VD3 into rigid anionic liposomes has proven to be relatively inefficient using traditional liposome preparation methods (Figure 5). Interestingly, we observed that the loading efficiency of VD3 into DSPC:DSPG:Cholesterol liposomes increases dramatically from 1.9% (± 0.02) to 63.4% (± 3.4) when the formulations are prepared using the staggered herringbone micromixer compared to the lipid film hydration method. The increased in loading efficiency was not accompanied by a change in liposome morphology as assessed by TEM neither aggregates of VD3 could be found in the TEM microscopy images. This data indicates that our microfluidics-based method is an excellent tool for encapsulation of both a wide range of peptide antigens as well as a tolerogenic adjuvant like VD3. Importantly, we show that preparation on rigid liposomes with microfluidics does not compromise its previously reported non-inflammatory uptake by dendritic cells and does not lead to dendritic cells activation. Therefore, the presented microfluidics method may be very useful for the production of liposomal formulations to induce immune tolerance.

Thus, in conclusion, here we describe the use of an off-the-shelf staggered herringbone micromixer for the preparation of highly rigid liposomal formulations in a size-tuneable and scalable manner. This system presents important advantages in terms of loading efficiency of the tolerogenic molecule VD3 compared to the

gold-standard lipid film hydration method. Highly rigid anionic liposomes can have intrinsic tolerogenic capacity and combined with tolerogenic molecules represent promising nanotherapeutics for the treatment of autoimmune and inflammatory diseases.

AUTHOR CONTRIBUTIONS

FLV conceptualised, designed, and carried out experiments, wrote the manuscript and prepared the figures. BS conceptualised and edited the manuscript. NAN performed uptake and DC activation experiments. MHT and RS performed part of the experiments. JAB, AK, WJ, ECJ provided valuable input for the experimental design and edited the draft manuscript.

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SUPPLEMENTARY DATA

Table S1. Flow conditions

Formulation#	Total Flow Rate (μL/min)	Flow Rate Ratio (Aqueous : Organic)	Aqueous flow rate (μL/min)	Organic flow rate (μL/min)
1	500	2:1	333.3	166.7
2		3:1	375	125
3		4:1	400	100
4		5:1	416.7	83.3

Table S2. Viscosity values and Refractive Index of the dispersant used in DLS method for samples measured before dialysis

Flow Rate Ratio (Aqueous : Organic)	% (v/v) of ethanol in formulation	Viscosity of dispersant (cP)	Refractive Index
2:1	33.4	2.4128	1.349
3:1	25	1.9970	1.344
4:1	20	1.8318	1.342
5:1	16.7	1.6398	1.339

Table S3. Peptide sequence and physicochemical properties of peptides used

Peptide name	Peptide sequence	Molecular weight (g/mol)	Net charge (at pH 7.4)	GRAVY
Peptide 1	INNQLTLDSNTKY	1565.69	0	-0.985
Peptide 2	INNQLTLDSNTKYFH	1850	0	-0.880
Peptide 3	IEGNLIFDPNNYLPK	1789	-1	-0.460
Peptide 4	SASYKADTVAKVQG	1466.6	1	-0.357
Peptide 5	LSASYKADTVAKVQG	1579.76	1	-0.080
Peptide 6	LKFIIPSPKRPVK	1564.96	4	-0.154
Peptide 7	IERYEVDQQIQVL	1674.86	-2	-0.431