Title: Microfluidic-controlled manufacture of liposomes for the solubilisation of a poorly water soluble drug.

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Abstract

Besides their well-described use as delivery systems for water-soluble drugs, liposomes have the ability to act as a solubilizing agent for drugs with low aqueous solubility. However, a key limitation in exploiting liposome technology is the availability of scalable, low-cost production methods for the preparation of liposomes. Here we describe a new method, using microfluidics, to prepare liposomal solubilising systems which can incorporate low solubility drugs (in this case propofol). The setup, based on a chaotic advection micromixer, showed high drug loading (41 mol%) of propofol as well as the ability to manufacture vesicles with at prescribed sizes (between 50 to 450 nm) in a high-throughput setting. Our results demonstrate the ability of merging liposome manufacturing and drug encapsulation in a single process step, leading to an overall reduced process time. These studies emphasise the flexibility and ease of applying lab-on-a-chip microfluidics for the solubilisation of poorly water-soluble drugs.

1 Introduction

The delivery of drugs by liposomes was first described in the 1970s by Gregoriadis (Gregoriadis and Ryman, 1971) and there is now a range of clinically approved liposome-based products that improve the therapeutic outcome for patients. Whilst liposomes are commonly considered for the delivery of aqueous soluble drugs, they are also well placed to act as solubilisation agents for drugs with low aqueous solubility. This is of considerable interest given that more than 40% of all new chemical entities in discovery have low solubility and subsequent issues in bioavailability (Savjani et al., 2012; Williams et al., 2012). The encapsulation of low solubility drugs into the bilayer of liposomes allows not only for their solubilisation in an aqueous media, but furthermore can offer protection from degradation and control over the pharmacokinetic drug distribution profile and improved therapeutic efficacy.

When solubilising drug within the liposomal bilayer, drug incorporation and release rates has been shown to depend on the properties of the drug, the composition of the liposomes, the lipid choice and concentration (Ali et al., 2010; Ali et al., 2013; Mohammed et al., 2004). For example, the log P and molecular weight are often considered to impact on bilayer loading, and studies have shown that molecular weight may play a dominant role (Ali et al., 2013). When considering the design of liposomes, there are a range of parameters that impact on bilayer loading efficacy. For example, we have previously shown that increasing the bilayer lipophillic volume (by adopting longer alkyl chain lipids within the liposomes) increases the loading ability of liposomal systems (Mohammed et al., 2004; Ali et al., 2013). Similarly, incorporation of charged lipids within the liposomal system may also impact on bilayer loading through electrostatic repulsion of drugs with like-charged liposomal bilayers (Mohammed et al., 2004). Incorporation of cholesterol, whilst stabilising the liposomes was also shown to inhibit bilayer drug loading (Ali et al., 2010) due to the space-filling action of cholesterol in the liposomal bilayer. By increasing the orientation order of the phospholipid hydrocarbon chains, cholesterol decreases bilayer permeability. Indeed, the presence of cholesterol in liposomes solubilising propofol was shown to shift the drug release profile from zero-order

(when no cholesterol was present) to first order (when 11 to 33 mol% of cholesterol was incorporated). This maps to the idea that without cholesterol the bilayer can be thought of as more 'porous' in nature compared with the more condensed and less permeable cholesterol-containing liposome bilayers (Ali et al., 2010).

However, whilst a wide range of studies have looked at the effect of formulation parameters on the application of liposomes as solubilising agents, more focus is required into making liposomes a cost-effective solubilising agent. Recent advances in lab-on-a-chip based tools for process development has already lead to microfluidic-based methodologies in drug development (Dittrich and Manz, 2006; Weigl et al., 2003; Whitesides, 2006). Indeed, microfluidics-based methods (which exploit controlled mixing of streams in micro-sized channels) have been described for the manufacture of liposomes and lipid nanoparticles (van Swaay, 2013). Liposome formation by microfluidics primarily depends on the process of controlled alterations in polarities throughout the mixer chamber, which is followed by a nanoprecipitation reaction and the self-assembly of the lipid molecules into liposomes. Generally, two or more inlet streams (lipids in solvent and an aqueous phase) are rapidly mixed together and flow profiles in the chamber itself are of low Reynolds numbers and categorized as laminar. Using microfluidic systems a tight control of the mixing rates and ratio between aqueous and solvent streams is achieved, with lower liquid volumes required, which facilitates process development by reducing time and development costs. The systems are designed with the option of high-throughput manufacturing and are generally considered as less harsh compared to conventional methods of liposome manufacturing that are based on mechanical disruption of large vesicles into small and unilamellar ones (Wagner and Vorauer-Uhl, 2011). Within the range of microfluidic mixing devices, we use a chaotic advection micromixer, a Staggered Herringbone Micromixer (SHM). The fluid streams are passed through the series of herringbone structures that allow for the introduction of a chaotic flow profile, which enhances advection and diffusion. A chaotic advection micromixer, as well as flow focusing methods, were shown to allow for scalability, associated with defined vesicle sizes (Belliveau et al., 2012;

Jahn et al., 2007). The method based on chaotic advection was shown to reproducibly generate small unilamellar liposomes (SUV) with tight control of the resulting liposome sizes at flow rates as high as 70 mL/min in a parallelized mixer-setup. We have previously shown that microfluidics can be used to produce cationic liposomal transfection agents (Kastner et al., 2014), where design of experiments and multivariate analysis revealed the ratio between aqueous and solvent phase having a strong relevance for the formation of size-controlled liposomes. Within this study, we have exploited microfluidics to develop a high-throughput manufacturing process to prepare liposomes solubilising drug within their bilayer (Figure 1).

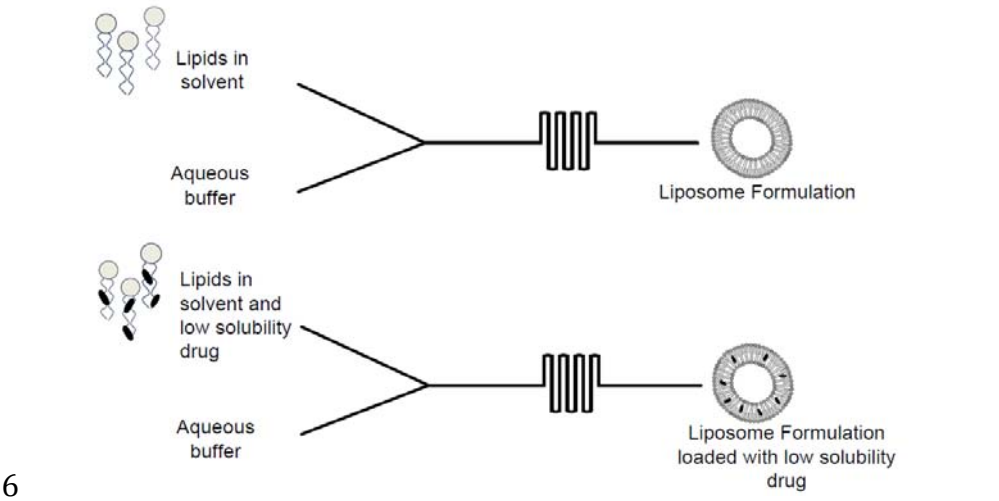


Figure 1: Schematic depiction of the liposome formation process based on the SHM design, a chaotic advection micromixer for (A) empty liposomes, (B) drug loaded liposomes and (C) chamber layout.

2 Materials and Methods

2.1 Materials

Egg Phosphatidylcholine (PC) and Cholesterol were obtained from Sigma-Aldrich Company Ltd., Poole, UK. Ethanol and methanol were obtained from Fisher Scientific UK, Loughborough, UK. TRIS Ultra Pure was obtained from ICN Biomedicals, Inc., Aurora, Ohio. Propofol (2,6-Bis(isopropyl)phenol) and 5(6)-Carboxyfluorescein (CF) was obtained from Sigma-Aldrich Company Ltd., Poole, UK.

2.2 Micromixer design and fabrication

The micromixer was obtained from Precision NanoSystems Inc., Vancouver, Canada. The mixer contained moulded channels which were 200 μm x 79 μm (width x height) with herringbone features of 50 x 31 μm. 1 mL disposable syringes were used for the inlet streams, with respective fluid connectors to the chip inlets. Formulations using the micromixer were performed on a NanoAssemblr™ (Precision NanoSystems Inc., Vancouver, Canada) that allowed for control of the flow rates (0.5 to 6 mL/min) and the flow ratios (1:1 to 1:5, ratio between solvent:aqueous) between the respective streams.

2.3 Formulation of small unilamellar vesicles using microfluidics

Lipids (16:4 molar ratio of PC and Cholesterol, 8:1 w/w) were dissolved in ethanol. SUV were manufactured by injecting the lipids and aqueous buffer (TRIS 10mM, pH 7.2) into separate chamber inlets of the micromixer. The flow rate ratio (FRR) (ratio between solvent and aqueous stream) as well as the total flow rate (TFR) of both streams were controlled by syringe pumps, calibrated to the syringe inner diameter. FRR varied from 1:1 to 1:5 and TFR varied from 0.5 to 6 mL/min, extrapolated from previous reported methods applying a SHM design with a channel diameter of 200 μm (Kastner et al., 2014). The SUV formulation was collected from the chamber outlet and dialysed at room temperature against TRIS buffer (10mM, pH 7.2) for removal of residual solvent. The model drug of low aqueous solubility was propofol (2,6-Bis(isopropyl)phenol), previously shown to correspond to high encapsulation values in liposomal systems due to

its low molecular weight (Ali et al., 2013). To encapsulate propofol, the low solubility drug was dissolved with the lipids in ethanol (0.5 to 3mg/mL) and thereby liposome formation and encapsulation of the drug was performed simultaneously using the micromixer method.

2.4 Lipid film hydration and sonication

Multilamellar vesicles (MLV) were prepared using the lipid film hydration method (Bangham et al., 1965). Basically, lipids were dissolved in chloroform/methanol (9:1 v/v) and the organic solvent was subsequently removed by rotary evaporation under vacuum to form a dry lipid film which was flushed with N_2 to ensure removal of solvent residues. The lipid film was hydrated with TRIS buffer (10 mM, pH7.2) to form MLV. SUV were then formed via probe sonication (Sonirep150plus, MSE; 5 min at an amplitude of 5).

2.5 Measurement of particle characteristics

Characterisation of the liposomes included size measurements using dynamic light scattering (DLS) (Malvern NanoZS), reported as the z-average (intensity based mean particle diameter) for monomodal size distributions and the zeta potential using particle electrophoresis (Malvern NanoZS). Polydispersity (PDI) measurements (Malvern NanoZS) were used to assess particle distribution.

2.6 Quantification of drug concentrations

Quantification of propofol was performed by reverse phase HPLC (Luna 5µ C18, Phenomenex, pore size of 100A, particle size of 5 µm). Detector was UV/Vis, at 268 nm. The flow rate was constant at 1.0 mL/min throughout with a gradient elution from 5% B (Methanol), 95% A (0.1% Trifluoroacetic Acid (TFA) in water) to 100% B over 10 minutes. HPLC-grade liquids were used, sonicated and filtered. The column temperature was controlled at 35ºC. All analysis was made in Clarity, DataApex version 4.0.3.876. Quantification was achieved by reference to a calibration curve produced from standards (six replicates in ethanol) at concentrations from 0.01 to 1 mg/mL. The calibration curve had a linearity $R^2 \geq$

0.997, and all measurements were within the level of detection and level of quantification.

2.7 Determination of drug loading into liposomes

The amount of drug loaded into the bilayer was measured by determination of the residual amount of drug in the liposome bilayer after removal of non-entrapped drug by dialysis (sink conditions) against 1 L of TRIS buffer, 10mM pH 7.2 (3500 Da, Medicell Membranes Ltd., London, UK). The drug content was measured by HPLC as described in section 2.6. This protocol was validated by assessing the rate of propofol removal by dialysis.

2.8 Stability study

For the stability study, formulations of propofol-loaded SUV were stored at 4°C, 25°C and 40°C in pharmaceutical grade stability cabinets over 60 days (time point measurements at day 0, 7, 14, 21, 28 and 60). Samples were taken at these specific time points for measurement of particle characteristics (section 2.5) and drug loading (section 2.6). Samples were dialysed against 500 mL TRIS buffer (10 mM, pH7.2, sink conditions) at each time point to remove non-entrapped propofol. Propofol content remaining in the liposome formulation was assessed by HPLC as described in section 2.6.

2.9 Recovery of lipids and propofol

To assess the overall lipid and propofol recovery in the microfluidics method, the amount of lipid and propofol was measured by HPLC and expressed as % recovery compared to the initial amount of lipids or propofol available in the stock. The HPLC method was the same as described section 2.6, and lipids were quantified by an evaporative light scattering (ELS) detector (Sedere, Sedex 90), set at 52°C and coupled to the HPLC.

2.10 Freeze Fracturing Imaging

Two microlitres of liposome suspension were placed in a ridged gold specimen support and frozen rapidly by plunging into a briskly stirred mixture of

propane:isopentane (4:1) cooled in a liquid nitrogen bath. Fracturing, with a cold knife, and replication were performed in a Balzers BAF 400D apparatus under conditions similar to those described previously for freeze-fracture of liposomes (Forge et al., 1978; Forge et al., 1989). The replicas generated were floated off on water, cleaned in domestic bleach diluted 1:1 in distilled water, and then washed several times in distilled water before mounting on grids for electron microscopy. The replicas were viewed in a JEOL 1200EXII transmission electron microscope operating at 80kv and digital images collected with a Gatan camera. Images of the freeze-fractured samples are presented in reverse contrast so that shadows appear black. Fracturing imaging was performed by Prof. Andrew Forge at UCL Ear Institute, London, UK.

2.11 Drug release study

The in-vitro release rate of the drug was determined by incubating the drug-loaded liposomes in 1 L TRIS buffer (10mM, pH 7.2) after removal of the non-incorporated drug, at 37°C in a shaking water bath (150 shakes/min). Three independent formulations of drug-loaded liposomes made by the microfluidics method (TFR 2 mL/min, FRR 1:3) and standard lipid film hydration followed by sonication were incubated (3 mL per formulation) and samples of 200 µL were withdrawn at time intervals of 0.5 h, 1 h, 2 h, 4 h, 8 h and 16 h. Drug quantification was performed as described in section 2.6 and expressed as % cumulative release relative to the initial amount of drug encapsulated.

2.12 Incorporation of an aqueous marker within liposomes

To validate the formulation of liposomes, the presence of an aqueous core within the nanoparticles manufactured was verified by including and imaging of an aqueous fluorescent dye. Liposomes were manufactured as described in section 2.3 and 2.4 with 1 mM Carboxyfluorescein (CF) included in the aqueous buffer (TRIS, 10 mM, pH 7.2). Liposomes with entrapped CF were separated from un-entrapped dye by dialysis over night against 1 L fresh TRIS buffer, pH 7.2. Liposomes were imaged under a confocal microscope SP5 TCS II MP, Leica

Microsystems, Leica TCSSP5 II, 63x objective (HCX PLAPO 63x/1.4-0.6 oil CS). Images were taken by Charlotte Bland, Aston University, ARCHA facility.

2.13 Statistical tools

If not stated otherwise, results were reported as mean ± standard deviation (SD). One- or two-way analysis of variance (ANOVA) was used to assess statistical significance, followed by Tukeys multiple comparing test and t-test was performed for paired comparisons. Significance was acknowledged for p values less than 0.05 (marked with *). All calculations were made in GraphPad Prism version 6.0 (GraphPad Software Inc., La Jolla, CA).

3 Results and discussion

3.1 Influence of the flow rate ratio of aqueous and solvent stream on liposome size

The increase in polarity throughout the chamber drives the formation of small unilamellar liposomes (SUV) in milliseconds of mixing. For their formation, the rate of mixing as well as the ratio of aqueous to solvent stream has been anticipated as crucial factors. The formation of the liposomes is based on a nanoprecipitation reaction, where supersaturation occurs and the liposomes are formed by self-assembly after aggregation of the lipid molecules. The initial aim of this work was to assess the formation of liposomes by microfluidic mixing and assess the efficacy of this system to act as a solubilising agent. Therefore, liposomes were prepared from PC and Cholesterol (16:4 molar ratio, 8:1 w/w) at different total flow rates (TFR) and flow rate ratios (FRR) and the size, polydispersity and zeta potential were measured.

Liposomes formed at low flow rate ratio (1:1) showed the largest size of around 450 nm; increasing the flow rate ratio resulted in smaller liposomes (around 40 - 50 nm) at constant flow rates of 2 mL/min (TRIS, 10 mM, pH7.2) (Figure 2A). However, increasing the flow rate ratio increased polydispersity (to a maximum of 0.4; Figure 2B). Liposomes prepared at a flow rate ratio of 1:3 are shown in

Figure 2C, demonstrating their small nature, with average sizes of the vesicles in agreement with average vesicle diameters obtained by particle sizing via dynamic light scattering (~40 nm). In contrast, the smallest vesicle size of a comparable formulation achievable via probe sonication with this lipid formulation was 100 nm in size at PDIs of 0.3 (data not shown). To verify the formation of liposomes, rather than micelles, the liposomes made by the microfluidics method were prepared encapsulating an aqueous fluorescent dye, carboxyfluorescein (CF, 1 mM), which was included in the aqueous phase during liposome manufacturing by microfluidics and lipid film hydration. After removal of the free CF by dialysis overnight, the remaining dye entrapped in the particles was visualized by confocal microscopy. Bright green fluorescent cores visible in the particles manufactured by the microfluidics method (Figure 2D) were in line with images obtained from liposomes manufactured with the lipid film hydration method (images not shown); which confirms the presence of aqueous cores and the formation of liposomes in the novel microfluidics method.

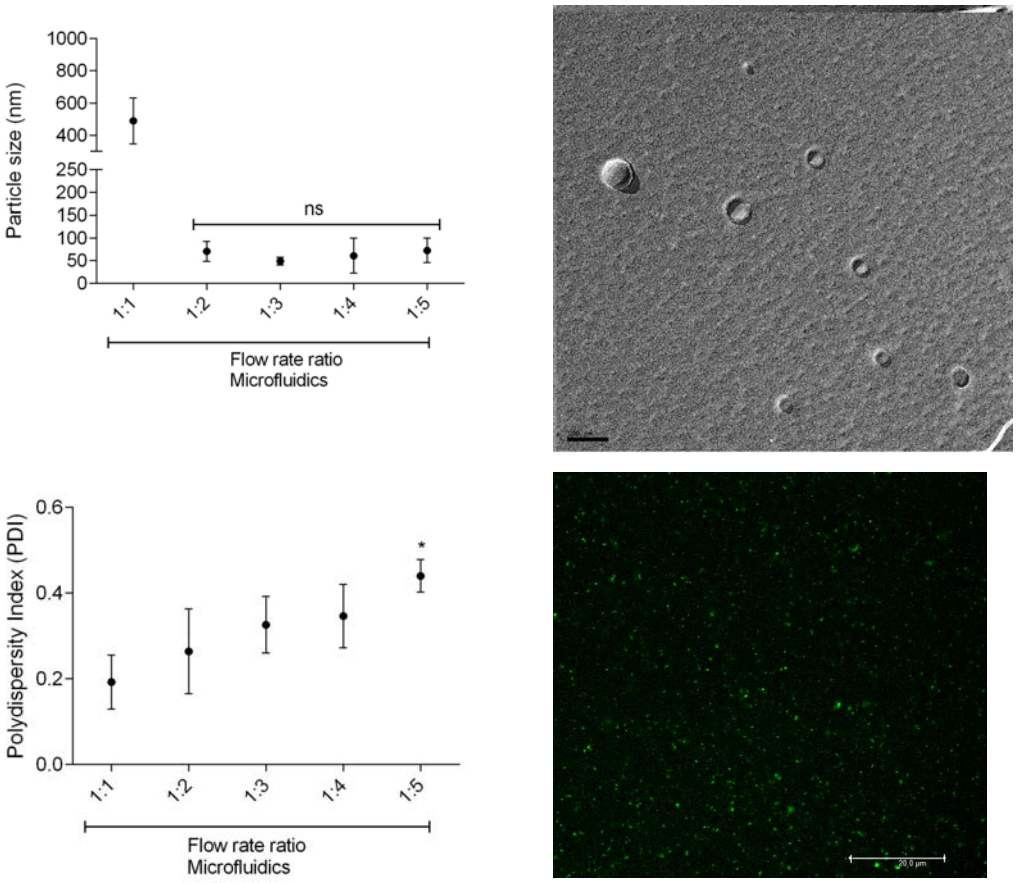


Figure 2: Liposome size (A) and polydispersity (B) of vesicles formulated with microfluidics method at increasing flow ratios. ns = not significant ($p>0.05$), * denotes statistical significance ($p<0.05$) in comparison to FRR 1:1 (C) Freeze fracturing electron microscopy images for empty liposomes manufactured with the microfluidics method. Bar represents 100 nm. (D) Fluorescent microscope images of liposomes manufactured with the microfluidics method, carboxyfluorescein was encapsulated within the aqueous core of the vesicles as a control for the manufacturing of bilayer liposomes. Bar represents 20 μm.

These impact of flow rate ratio on vesicle size are in agreement with previous work showing that the increase in FRR reduces the resulting size of the liposomes (Jahn et al., 2010; Kastner et al., 2014; Zook and Vreeland, 2010). A correlation between higher flow rate ratios and smaller liposome particles has been reported using liposomes composed of 1-palmitoyl, 2-oleoyl phosphatidylcholine (POPC), cholesterol and the triglyceride triolein, which resulted in the production of vesicular structures with sizes ranging from 140 nm to 40 nm dependent on the FRR chosen and triglyceride emulsions between 20– 50 nm size with nonpolar cores (Zhigaltsev et al., 2012). The overall lower amount of residual solvent present at higher FRR employed decreases the particle fusion (Ostwald ripening), which leads to the formation of smaller particles (Zhigaltsev et al., 2012). The increase in polydispersity may be a result of increased dilution at higher FRR reducing the rate of diffusional mixing within the micromixer as noted in previous studies applying a SHM mixer for liposome manufacturing (Kastner et al., 2014). With diffusion being proportional to the lipid concentration, increasing FRR is effectively reducing the lipid concentration, thus reducing the rate of diffusion, leading to partly incomplete nucleation and a lower rate of liposome formation inside the micromixer (Balbino et al., 2013b). Overall, these findings demonstrate that a FRR of 1:2 to 1:4 result in liposomes of the smallest size and polydispersity. The dilution factor (due to flow ratios chosen involved in the SHM method) is overall lower compared to ratios employed in the flow-focusing method, which can reach up to

60 (Jahn et al., 2010; Jahn et al., 2007; Jahn et al., 2004). Furthermore, the SHM method enhances the diffusional mixing due to the herringbone structures on the channel wall (Stroock et al., 2002), which results in an enhanced mixing profiles compared to the flow-focusing technique.

3.2 Influence of flow rate on throughput and particle characteristics

To assess the ability of the system as a potential high-throughput manufacturing method for liposomal solubilisation systems, we increased the total flow rate 3-fold whilst maintaining the ratio between aqueous and solvent stream constant. Liposome size was shown to be independent of the applied flow rate, with no significant change in vesicle size (Figure 3A), pdi (Figure 3B) and zeta potential (-3±2mV; data not shown). These results support the suitability of microfluidics manufacturing as a high throughput method with liposome characteristics being maintained constant whilst increasing the total flow rate in the system. Our results also confirm that the flow rate ratio used in the system is the most crucial variable on liposome size, which has previously been demonstrated with other systems (Balbino et al., 2013a; Balbino et al., 2013b; Jahn et al., 2007; Jahn et al., 2004; Kastner et al., 2014). The scalability of the microfluidics method has been suggested by Belliveau et al. 2013, by parallelization of the mixer chamber. Scalability and increase in throughput together demonstrate the industrial applicability comparable with scale-up options available (Wagner and Vorauer-Uhl, 2011).

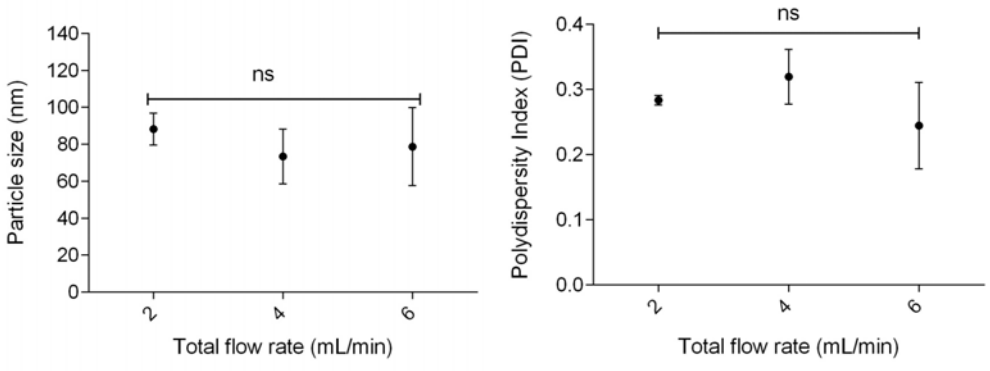
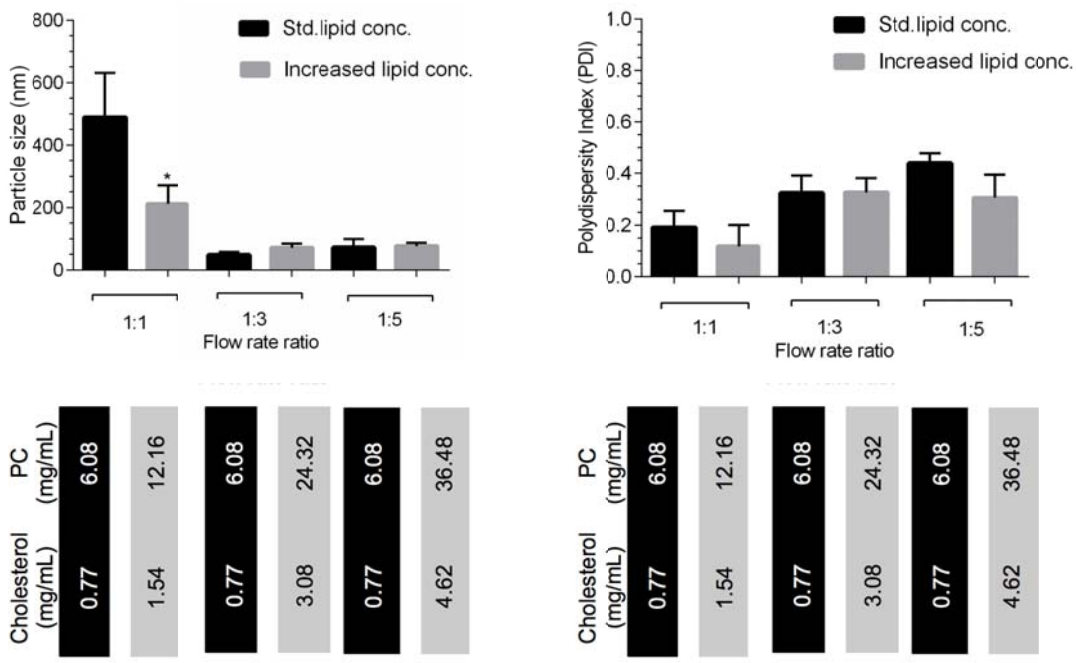


Figure 3: Liposome size (A) and polydispersity (B) of vesicles formulated with microfluidics at increasing flow rates and constant flow ratio of 1:3, n = 3, ns = not significant ($p>0.05$).

As shown, the increase in FRR is the main contributing factor governing liposome size (Figure 2A). Nevertheless, an increase in FRR will inevitably lead to dilution and lower liposome concentrations in the final liposome suspension produced. A subsequent concentration process based on filtration (Pattnaik and Ray, 2009), chromatography (Ruysschaert et al., 2005) or centrifugation adds additional processing time. Therefore, to circumvent this additional process step, we counteracted the dilution of the lipids at higher FRR by increasing initial lipid concentrations introduced to the micromixer at the desired FRR. Through this method, liposomes were manufactured at up to 6 fold higher concentrations. Increased lipid concentrations at FRR of 1:3 and 1:5 did not significantly ($p>0.05$) influence size and polydispersity compared to the standard lipid concentration (Figure 4A and B), whereas at a FRR of 1:1 a significant ($p<0.05$) decrease in vesicle size was observed (Figure 4A). At this lower FRR, the higher lipid concentrations may again decreasing particle fusion leading to the formation of smaller particles (Zhigaltsev et al., 2012). Nevertheless, this setup allows to increase the final liposome concentration according to the FRR chosen without adversely changing resulting vesicle size or polydispersity for the smallest vesicle sizes obtained at higher FRR (Figure 4A and B respectively), due to the diffusional mixing process in the SHM design.



404 Figure 4: Increase in the lipid concentration in the ethanol stock to circumvent
405 the dilution effect at flow ratios of 1:1, 1:3 and 1:5 for (A) liposome size, *
406 denotes statistical significance ($p<0.05$) in comparison to FRR 1:1 for the
407 standard lipid concentration and (B) polydispersity with respective
408 concentration of PC and Cholesterol in the inlet stream, n = 3.

405

406

408 **3.3 Drug loading studies: The effect of drug encapsulation by the liposome**
409 **manufacturing method**

416 So far, we have shown that the microfluidics method allows for size-controlled
417 and rapid synthesis of liposomes. To consider the applicability of this method to
418 be used for a high-throughput production of liposomes as solubilising agents the
419 loading capacity of the formulation was considered. Based on the optimisation
420 studies shown in Figure 2, propofol was solubilised within liposomes prepared
421 at a FRR of 1:3 and a TFR of 2 mL/min. The particle characteristics and drug
422 loading efficiency (mol%; Figure 5A) was determined at propofol concentrations
423 ranging from 0.5 to 3 mg/mL (effective concentration in the solvent stream).

417

419 Using a propofol concentration of 1 mg/mL in the solvent stream showed high
420 drug loading (~50 mol%), combined with particle size of ~50 nm and a low

polydispersity (Figure 5A). Particle size and polydispersity increased notably (ca. 600 nm and 0.8 respectively) at the highest propofol concentration (3 mg/mL in the solvent stream, giving a loading of ~25mol%, Figure 5A), suggesting the liposome system may have become saturated or destabilised at high propofol concentrations (drug-to-lipid ratio 1.72 mol/mol). Based on this, subsequent studies adopted a propofol concentration at 1 mg/mL in the solvent stream for all performed encapsulation studies.

The drug encapsulation was further investigated as a function of FRR in the microfluidics method. Propofol encapsulation (mol%) in liposomes prepared at FRR 1:1, 1:3 and 1:5 remained at approximately 50 mol% with no statistical difference. However this was significantly higher ($p < 0.0001$) than drug loading in liposomes prepared via sonication (15 mol%; Figure 5B). The drug loading efficiency of liposomes prepared by sonication is in line with previous reported propofol encapsulation (Ali et al., 2013). Furthermore, drug encapsulation did not alter vesicle size or polydispersity (Figure 5A) and vesicle sizes obtained by dynamic light scattering were verified by freeze fracturing images (Figure 5D). This higher drug loading may be a result of the highly efficient mixing processes occurring during microfluidics that favours incorporation of propofol within the bilayers in the same process as the vesicles form. Indeed, the here presented method allows to achieve a propofol encapsulation of ~50 mol%, which represents a total propofol amount of ~300 mg/mL in the final liposome formulation, representing a 2000-fold increase to the reported aqueous solubility of propofol, 150 μg/mL (Altomare et al., 2003).

Size (nm)	40 ± 2	48 ± 2	613 ± 360
PDI	0.03 ± 0.02	0.17 ± 0.01	0.8 ± 0.28

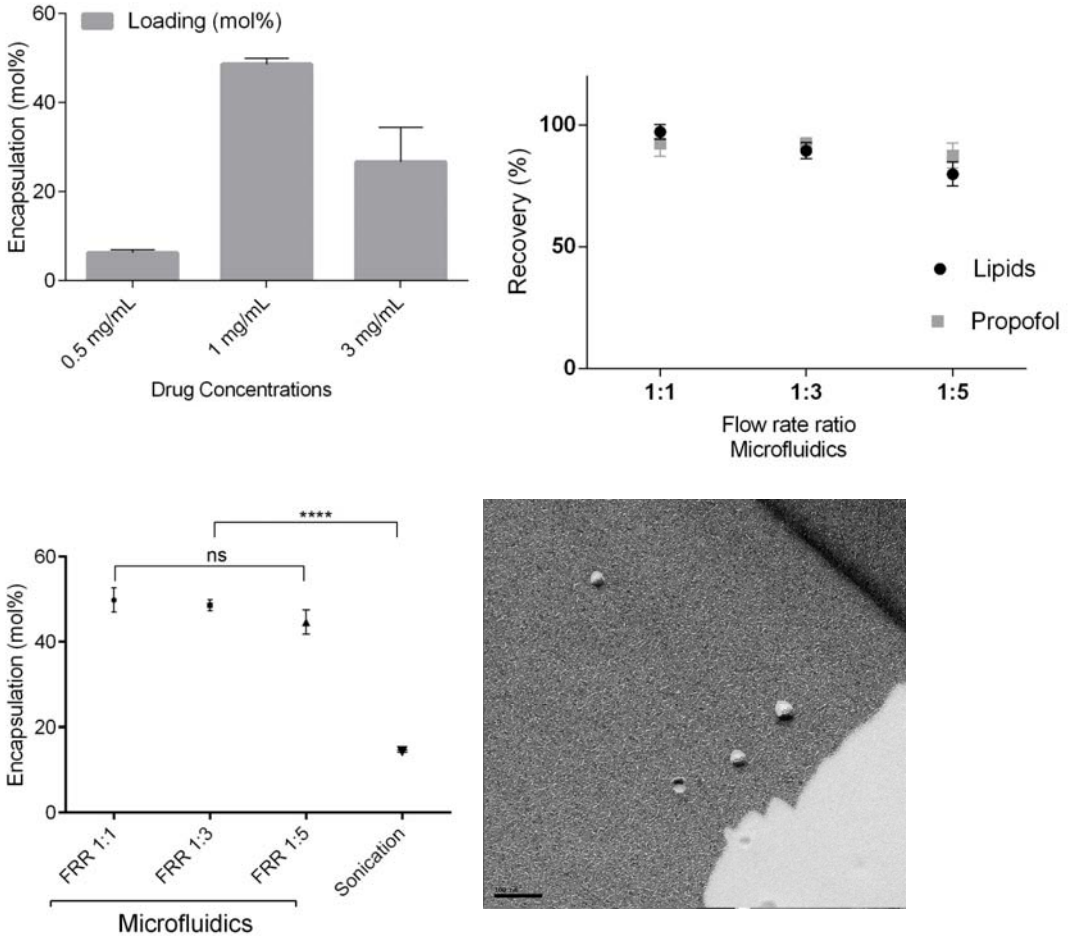


Figure 5: (A) Effect of drug concentrations in the ethanol inlet stream (0.5, 1 and 3 mg/mL) on encapsulation efficiency (mol%), particle size and polydispersities at a flow ratio of 1:3.. (B) Encapsulation efficiency (mol%) of liposomes formed with the microfluidics method at flow ratios of 1:1, 1:3 and 1:5 compared to the encapsulation efficiency using the sonication method. Results are average out of triplicate formulations and measurements. ns = not significant ($p>0.05$), * denotes statistical significance ($p<0.00001$) in comparison to microfluidics-based samples. (C) Recovery of lipids and propofol in the microfluidics method at different flow ratios. Results are expressed as % compared to the initial lipid and propofol amount present (n = 3). (D) Freeze fracturing electron microscopy

images for liposomes loaded with the low solubility model drug (propofol) manufactured with the microfluidics method. Bar represents 100 nm

To consider, drug release profiles, the *in-vitro* release of propofol encapsulated in liposomes by microfluidics was monitored at 37°C over 16 h. Liposomes formed with the microfluidics method had a significant higher drug encapsulated at the start of the release study (~55 mol%) compared to those vesicles formed by sonication (20 mol% drug encapsulation). However, relative to initial loading, an initial release of ca 40% was observed at 1 h for both formulations, followed by a continuous release of 90% of the encapsulated drug was observed over 8 h (Figure 6). Whereas the fatty alcohol alkyl chain length was shown to affect the release profile of encapsulated propofol (Ali et al., 2013), here the method of liposome manufacturing was shown to mainly affect the amount of drug incorporated into the liposomes, without altering the release profile of the encapsulated drug against sink conditions. Previous we have shown that solubilisation of propofol in phosphatidylcholine liposomes followed a zero-order release kinetics, where the incorporation of a higher amount of cholesterol shifted the release rates towards a first-order release model (Ali et al., 2010), implying that the release kinetics itself are mainly dominated by the lipid composition and physicochemical characteristics rather than the method of liposome manufacturing. This may prove advantageous in the development of an IV formulation; the pharmacokinetic release profile of propofol has been studies previously in a colloidal dispersion between 20-100 nm (Cai et al., 2012), where rapid distribution of propofol compared to the commercial product Diprivan® highlighted the need on the development of new techniques for the encapsulation of low solubility drugs.

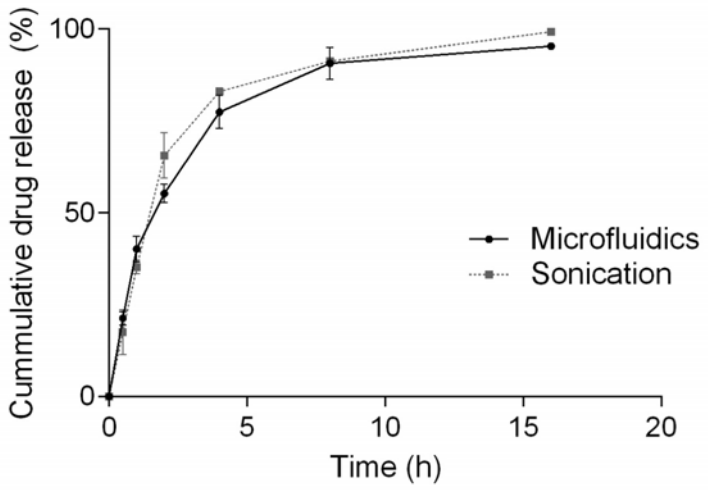


Figure 6: Effect of manufacturing method to the drug release of propofol from liposomes. Results show the cumulative drug release profile from formulations manufactured with the standard lipid film hydration / sonication method and microfluidics and represent percentage cumulative release of initially entrapped propofol, expressed as the means of three experiments ± SD.

It is important to verify both lipid and drug recovery when using the microfluidics method, to ensure cost-effectiveness and that lipid and drug concentrations remain locked at the ratio initially designed prior to formulation. To date, the quantification of lipids is mainly dominated by time intensive assays like mass spectrometry (Moore et al., 2007). Here, we introduce a simple and robust method of lipid quantification based on evaporative light scattering (ELS) detection and HPLC separation. We coupled an ELS detector downstream a HPLC separation method, which allowed for quantification of any solids in the eluate with a lower volatility than the mobile phase. Microfluidics based liposomal-drug formulations showed good recovery of the drug (88 - 92%; Figure 5C), independent of the FRR. Similarly, lipid recovery was high at FRR of 1:1 and 1:3 (97% and 89%; for FRR 1:1 and 1:3 respectively; Figure 5C). A significant drop (79%; $p<0.01$) in lipid recovery was noted at a flow ratio of 1:5, suggesting that higher FRR employed in the microfluidics method may impede lipid recovery due to enhanced dilution in the chamber. Nevertheless, the smallest vesicle size (~50nm) can be obtained at a FRR of 1:3 (Figure 2A) and any further increase in

FRR will not benefit the formulation (size, pdi and drug encapsulation). Based on this, we chose the FRR 1:3 for a long-term stability study.

3.4 The effect of manufacturing methods on liposome stability and drug encapsulation over 8 weeks

The SHM method was previously investigated for the encapsulation of a highly soluble drug, with approximately 100% loading efficiencies being reported using doxorubicin as a model drug (Zhigaltsev et al., 2012); the authors demonstrated high drug retention of encapsulated drug with liposomes stored at 4°C over the course of eight weeks (Zhigaltsev et al., 2012). Following the assessment that liposomes manufactured by the microfluidics method yields significant higher encapsulation of propofol, similarly we performed an eight-week stability study to verify the integrity of the vesicles at different storage temperatures. Vesicles were prepared using microfluidics as described above, and the initial amount of propofol encapsulated was determined after removal of free drug by dialysis. Vesicles were stored at 4°C, 25°C/60%RH and 40°C/75%RH (standard ICH temperatures) in pharmaceutical grade stability cabinets and the formulations made by the sonication method were stored at 25°C/60%RH (Figure 7, Table 1), acting as the control method. The control liposomes formed by sonication showed good stability in terms of size retention over the course of the study. Similarly, for liposomes prepared using microfluidics, vesicle size remained unaffected after storage over 8 weeks at 4°C and 25°C. In contrast, liposomes stored at 40°C significantly increase in size from initially 55 nm to 120 nm (Figure 7A), with no notable affect to polydispersity, suggesting the liposome population as a whole has changed in size rather than a sub-set of the vesicles (Table 1).

Table 1: Polydispersity at different storage conditions for 8 weeks. Results are mean out of triplicate formulations and measurements.

Day	0	7	14	21	28	60
Microfluidics						
4°C	0.403 ± 0.02	0.286 ± 0.01	0.282 ± 0.01	0.295 ± 0.01	0.261 ± 0.01	0. 305 ± 0.01

25°C	0.403 ± 0.02	0.295 ± 0.01	0.279 ± 0.01	0.301 ± 0.04	0.302 ± 0.03	0.266 ± 0.03
40°C	0.403 ± 0.02	0.254 ± 0.001	0.121 ± 0.02	0.119 ± 0.001	0.129 ± 0.01	0.221 ± 0.01
Sonication						
25°C	0.656 ± 0.02	0.652 ± 0.02	0.522 ± 0.15	0.658 ± 0.049	0.552 ± 0.04	0.505 ± 0.06

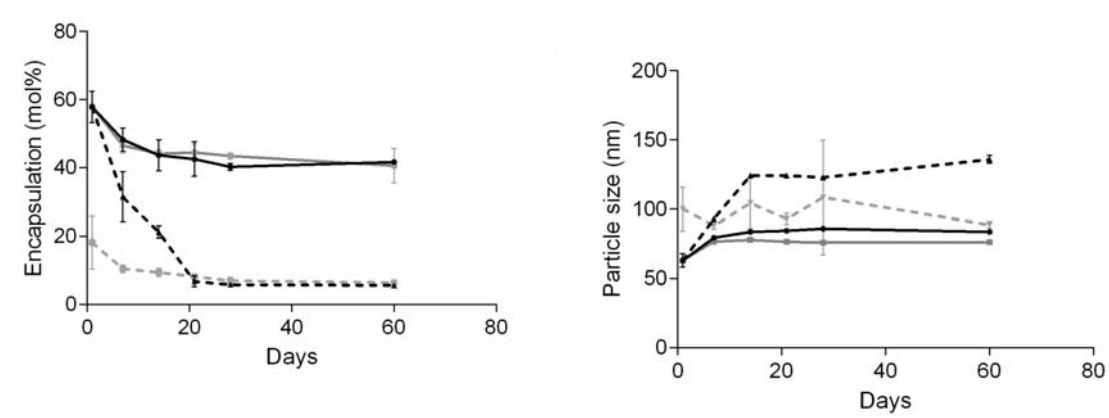


Figure 7: Size (A) and drug encapsulation (mol%) (B) at different storage conditions over 8 weeks. Results are mean of triplicate formulations and measurements.

Minor (but not significant) drug loss from the liposomes was detected for the formulations at 4°C and 25°C after the first 7 days of storage (Figure 7B), after which the formulations remained stable with final drug encapsulation values of 41±1 mol% and 41±4 mol% at 4°C and 25°C storage conditions respectively (Figure 7B). Similarly, with liposomes formulated using sonication showed and initial drug loss when stored at 25°C/60%RH which then plateaued out (Figure 7B). Notable drug loss from the microfluidic systems was only seen when they were stored at elevated temperatures with the formulation stored at 40°C showing almost complete drug loss over the course of the stability study, with only 5±1 mol% drug remaining encapsulated after 8 weeks, similar to the final drug encapsulated in the sonicated liposomes which were stored at 25°C/60%RH (Figure 7B). Overall, vesicles produced with the microfluidics method were smaller with a lower polydispersity than those obtained by lipid film hydration / sonication. The vesicles manufactured by sonication maintained their size around 100±20 nm throughout the stability study (stored at 25°C) as well as their polydispersity (Table 1). Results suggest that the method of

manufacturing mainly impacts the drug encapsulation rather than the physical properties (size, pdi, zeta potential). Stability of the formulations is crucial and these results demonstrate that liposomes formed by the microfluidics method remain over two months at conditions of 4 and 25°C.

3.5 Conclusion

Here, for the first time, we have demonstrated a high-throughput, robust method of preparing size-controlled liposomes as solubilising agents using microfluidics. These liposomes have well defined, scalable, process controlled, physico-chemical attributes demonstrating this method is suitable for pre-clinical and clinical production of liposomes. Drug loading was shown to be in an applicable range for clinical application (Biebuyck et al., 1994). Furthermore, using this novel method, liposome manufacturing and drug encapsulation are processed in a single process step, circumventing an additional drug loading step downstream, which notably reduces the time for production of stable drug-loaded vesicles of specified physico-chemical characteristics.

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688