Title: Microfluidics based manufacture of liposomes simultaneously entrapping hydrophilic and lipophilic drugs.

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Keywords: Liposomes, microfluidics, water soluble drugs, poorly soluble drugs, bilayer loading, manufacturing.

Abstract

Despite the substantial body of research investigating the use of liposomes, niosomes and other bilayer vesicles for drug delivery, the translation of these systems into licensed products remains limited. Indeed, recent shortages in the supply of liposomal products demonstrate the need for new scalable production methods for liposomes. Therefore, the aim of our research has been to consider the application of microfluidics in the manufacture of liposomes containing either or both a water soluble and a lipid soluble drug to promote co-delivery of drugs. For the first time, we demonstrate the entrapment of a hydrophilic and a lipophilic drug (metformin and glipizide respectively) both individually and in combination using a scalable microfluidics manufacturing system. In terms of the operating parameters, the choice of solvents, lipid concentration and aqueous:solvent ratio all impact on liposome size with vesicle diameter ranging from ~90 to 300 nm. In terms of drug loading, microfluidics production promoted high loading within ~100 nm vesicles for both the water soluble drug (20 - 25% of initial amount added) and the bilayer embedded drug (40 – 42% of initial amount added) with co-loading of the drugs making no impact on entrapment efficacy. However, co-loading of glipizide and metformin within the same liposome formulation did impact on the drug release profiles; in both instances the presence of both drugs in the one formulation promoted faster (up to 2 fold) release compared to liposomes containing a single drug alone. Overall, these results demonstrate the application of microfluidics to prepare liposomal systems incorporating either or both an aqueous soluble drug and a bilayer loaded drug.

1. Introduction

Since their discovery in the 1960s (Bangham and Horne, 1964) and first application as drug delivery systems (Gregoriadis and Ryman, 1971), liposomes continue to offer new opportunities to improve the delivery and targeting of a range of therapeutic agents, from small molecules through to large biologicals. Furthermore, thanks to the research of AT Florence, who first demonstrated the ability to formulate bilayer vesicles from non-ionic surfactants, also known as non-ionic surfactant vesicles (NISVs) or niosomes (e.g. (Azmin et al., 1985; Baillie et al., 1985; Uchegbu and Florence, 1995)), a range of other bilayer vesicles have been developed. For example, vesicles built from surfactant polymers (e.g. polymersomes (Okada et al., 1995)), cationic systems which can electrostatically bind DNA (e.g. lipoplexes (Felgner et al., 1987)), vesicles incorporating bile salts to improve stability (e.g. bilosomes (Conacher et al., 2001)), or virus components (e.g. virosomes (Almeida et al., 1975)).

However, despite the substantial body of research investigating their use, the translation of these bilayer vesicles into licensed products remains limited, with approximately 15 products currently approved, including the first generic version of liposomal doxorubicin hydrochloride (Lipodox). The approval of Lipodox by the US Food and Drug Administration (FDA) in priority review was aimed to ensure that provision of doxorubicin hydrochloride liposomal injection was not interrupted, despite supply shortages of the liposomal doxorubicin product Doxil (licensed for the treatment of ovarian cancer). These supply shortages started in 2011 when the FDA identified issues in the manufacturing site responsible for the production of Doxil.

In general, the manufacturing considerations of liposomal products can be considered a notable hurdle, given the cost and relative complexity of their production. In terms of characteristics and attributes to be considered for liposome drug products, these range from the physico-chemical properties of all the individual components (include the drug substance, the lipids and non-lipid components of the system) and the resulting liposomal product. Given that the pharmacological, toxicological and pharmacokinetic properties of the drug can be dictated by the liposomal product, quantification of the amount of drug incorporated and retained within the system must be defined. Furthermore, given that the pharmacokinetic profiles of the liposomal products are dictated by the liposomal physicochemical properties

(e.g. size, morphology, surface characteristics, liposome structure and integrity, net charge etc.), these should be characterised and defined. Indeed these are key critical quality attributes of a liposomal product and are often dictated by the method of manufacture.

Given the recent issues seen in the manufacture of liposome products and to facilitate the transition of more liposomal products from bench to clinic, it is important that new, low-cost and scalable manufacturing methods for liposomes and their related systems are developed. At the basic level, there are two main ways of forming liposomes: either to produce large vesicles and then employ size reduction methods (e.g. homogenisation, microfluidisation, high-sheer mixing and sonication), or alternatively bottom up methods, which promote the formation of small vesicles from individual lipid monomers. Whilst the production of large vesicles followed by size reduction is the commonly adopted method at the laboratory scale, such methods of liposome manufacture lack industrial scalability and encapsulation efficiencies are usually low.

In contrast, methods that exploit fluidic control to build liposomes from the bottom-up tend to offer more industrial applicability. For example, the ethanol injection method was the first one reported in the 1970s by Batzri and Korn (Batzri and Korn, 1973). Using this method, the formation of liposomes results from the rapid injection of lipids dissolved in ethanol into an aqueous buffer stream; the precipitation of the lipids leads to the formation of vesicles. This method is relatively simple and easy to scale, with the process considerations including the solubility of the lipids in the water-miscible solvent, rate of injection, and effective solvent removal post-processing. Recent variations on this method include the adoption of inkjet injection methods (Hauschild et al., 2005).

More recently, microfluidics has been considered for the formulation of liposomes (Jahn et al., 2007; Kastner et al., 2014; Kastner et al., 2015). The application of microfluidic tools for liposome manufacturing is based on the theory of a nanoprecipitation reaction resulting from rapid mixing at the nanolitre scale (Song et al., 2008; deMello, 2006). In contrast to the top-down methods for liposome manufacture, this nanoprecipitation can produce liposomes and nanoparticles in a one-step process (Bally et al., 2012), with no further disruption of the resulting liposomes required. The advantages of microfluidic-based technologies include enhanced control over processing conditions, offering reproducible and robust

manufacturing of uniform liposome size distributions and, by working at reduced volumes during development processes, costs can be reduced, whereas throughput is also increased (Carugo et al., 2016; Jensen, 2001; van Swaay and deMello, 2013; Weibel and Whitesides, 2006). Furthermore, variations in flow rate and flow rate ratios allows for the engineering of liposome-based systems in the range of 30 - 80 nm for small interfering RNA (siRNA) delivery (Belliveau et al., 2012; Zhigaltsev et al., 2012), DNA (Kastner et al., 2014) and low solubility drugs (Kastner et al., 2015). In the application of microfluidics for liposome manufacture, there are a range of parameters to be considered (Figure 1) and testing and optimisation of these parameters is important since they can impact on the critical product attributes of the liposomal systems (e.g. Kastner et al., 2014). Parameters to be considered range from input parameters – such as solvent selection, which can be influenced by lipid solubility – to manufacturing parameters – such as chip design, flow rate of solvents through the chip and the ratio they are mixed at, whilst temperature may also be a consideration (in the case of high-transition temperature lipids). In terms of the chip design, micromixers can be classified into active and passive mixers (Capretto et al., 2011). Passive micromixers require an input from an external energy source, e.g. pressure-driven, temperature-induced or ultrasonic-driven. In contrast, so called passive mixers do not require an additional external energy source to achieve mixing, but use the fluid flow and specially designed micro-structures that enhance diffusion and advection processes (Nguyen and Wu, 2004). In the production of liposomes, a range of chip designs have been tested, including a staggered herringbone micromixer based on patterns of grooves in the channel floor (Figure 1). The design introduces a chaotic flow in a microchannel by subjecting the fluid to repetitive series of a rotational flow profile, which is achieved by alteration of the grooves as a function of the axial position in the channel (Stroock et al., 2002).

Within our laboratories we have already demonstrated the use of microfluidics to formulate DNA-liposome complexes (Kastner et al., 2014) and incorporate low solubility drugs within the bilayer of the liposomes (Kastner et al., 2015); however, the passive incorporation of a hydrophilic drug has yet to be explored. Therefore, the aim of this current research is to build on this knowledge, and demonstrate the use of microfluidics to prepare sub-100 nm liposomes incorporating aqueous soluble drugs within their core. Furthermore, this study

investigate the preparation of liposomes co-entrapping both a hydrophilic and lipophilic drug within the same formulation to promote co-delivery of drugs.

2. Materials and methods

2.1. Materials

Egg phosphatidylcholine (PC), 1,2-dimyristoylphosphatidylcholine (DMPC), 1,2-diplmitoylphosphatidylcholine (DPPC), 1,2-disteroylphosphatidylcholine (DSPC) were purchased from Avanti Polar Lipids, Inc. (Alabaster, AL). Cholesterol was from Sigma-Aldrich Company Ltd. (Poole, UK). Glipizide, metformin HCl and Phosphate buffered saline (PBS) in tablet form (pH 7.4) were purchased from Sigma-Aldrich Company Ltd. (Poole, UK). All the solvents used in the analysis were of analytical grade and were purchased from Fisher Scientific UK (Loughborough, UK). Water used in the process of liposome preparation was of milli-Q grade.

2.2. Preparation of liposomes using microfluidics

To prepare liposomes, the NanoAssemblr^{TM} benchtop (Precision Nanosystems, Agronomy Rd, Vancouver) was used with a 300 micron Staggered Herringbone Micromixer. Briefly, the lipids at the appropriate ratio were dissolved in methanol. The aqueous buffer used in all studies was PBS, 10 mM, pH 7.4. The flow rate ratio (FRR) between the aqueous and solvent stream was varied from 5:1 to 1:1 (aq:solvent ratio) and the total flow rate (TFR) was varied from 5 to 15 mL/min. Through this method, liposome formation and incorporation of the drug(s) can be performed simultaneously by addition of the drug into the appropriate phase; within these studies, glipizide was dissolved in the solvent phase (1.1 mg/mL), whilst metformin was dissolved in PBS prior to microfluidic mixing. The liposome formulations were collected from the chamber outlet and dialysed at room temperature against PBS buffer for removal of residual solvent and non-loaded drug.

2.3. Liposome characterisation

Dynamic light scattering (DLS) was used to determine the size, reported as Z-average (based on intensity), and polydispersity index (PDI) of liposomes using Malvern NanoZS (Malvern Instruments, Worcestershire, UK). Particle size was measured in PBS diluted 1 in 300, pH 7.4, 25 ^{o}C. The zeta potential was also measured using the Malvern NanoZS; based on the particle electrophoresis principle in PBS, 1 mM, pH 7.4, 25 ^{o}C.

2.4. Removal of solvent and non-incorporated drug

3500-dalton dialysis tubing (Medicell membranes Ltd, London, UK) was used to remove residual solvent and non-entrapped drug from the liposomal suspension. Prior to use, the dialysis tubing was soaked under running water for two hours. Dialysis was performed using PBS (composition: phosphate buffer 0.01 M, 0.0027 M potassium chloride and 0.137 M sodium chloride, pH 7.4).

2.5. Quantification of lipid recovery

HPLC- ELSD (high performance liquid chromatography- evaporative light scattering detector) was used to quantify the lipid recovery of liposomes produced by microfluidics at a 3:1 FRR and 15 mL/min TFR. A Luna column (C18(2), 5 µm, dimensions 4.60 X 150 mm, pore size 100 Å) from Phenomenex (Macclesfield, UK) was used to detect the lipids. A 2 mL/min flow rate was used with a twenty minute elution gradient, composed of solvent A (0.1% TFA in water) and solvent B (100% methanol). During the first six minutes the gradient was 15:85 (A:B), at 6.1 minutes 0: 100 (A:B) and then back to the initial gradient of 15: 85 (A:B) from 15.1 to 20 minutes. The phospholipid and cholesterol lipids were analysed within the same run as they have different elution times. The lipid recovery was calculated as a percentage in comparison to the initial concentration of the stock solution.

2.6. Quantification of non-entrapped and entrapped drugs

Simultaneous quantification of metformin and glipizide (both liposome entrapped and non-entrapped) was performed using reversed-phase high performance liquid chromatography (RP-HPLC, Shimadzu 2010-HT, Milton Keynes, UK) connected with an ultra-violet detector at 233 nm to allow simultaneous quantification of both drugs. Isocratic elution was performed using mobile phase of acetonitrile:PBS (65:35, pH 5.75) at constant flow rate of 1.0 mL/minute, using a Luna column (C-18, 5µ, i.d. 150 X 4.6 mm) from Phenomenex (Macclesfield, UK). A calibration curve produced from linear standards was used as reference for the quantification of unknown. The calibration curved reported linearity (R^2) >0.995 and all measurements were within the level of detection and level of quantification. Drug incorporation is reported as % of initial amount used, and in all instances, overall recovery of both drugs was also determined from amount entrapped and non-entrapped drug and was between 90 to 110%.

2.7. Drug release study

The CE7smart USP-4 system (SOTAX AG, Switzerland) was used to create an incubating environment for the release of drug encapsulated within liposomes. PBS (pH 7.4) was used in a closed loop system and was circulated at constant temperature (37 ± 1°C) at a constant flow of 8.0 mL/minute. Samples were withdrawn at time intervals of 10, 30, 60, 90, 120, 180, 360, 540, 720 and 1440 minutes. Drug release was quantified using RP-HPLC (described in section 2.6.) and reported as % release relative to amount of drug entrapped within liposomes.

2.8. Cryo-TEM imaging of liposomes

All the samples were freshly prepared on the day of analysis. Empty, single and co-drug loaded liposomes were prepared using the method described before (section 2.2). A 3 µl aliquot of each sample was placed onto a pre-cleaned lacey carbon coated grid and flash frozen by plunging into liquid ethane cooled by liquid nitrogen. Samples were stored in liquid nitrogen and conveyed to a cryo-holder and observed under the electron microscope at liquid nitrogen temperatures. Grids were observed using Tecnai 12 G2 electron microscope (FEI, Eindhoven)

at 80 kV and the evaluation was performed in the magnification range of 40000 X to 135000 X.

2.9. Statistical tools

Unless stated otherwise, the results were calculated as mean ± standard deviation (SD). ANOVA followed by Tukey post hoc analysis was performed for comparison and significance was acknowledged for p values less than 0.05. All the calculations were made using Graphpad version-6 (GraphPad Inc., La Jolla, CA).

3. Results

3.1 Solvent selection and lipid concentrations in the manufacture of liposomes using microfluidics

When establishing the process of liposome manufacture using microfluidics, one of the initial input parameters to consider is the solvent selection. For appropriate mixing to occur, the solvent needs to be miscible with the aqueous phase. Other factors dictating this solvent selection are the lipid and drug compatibility and solubility in the selected solvent and aqueous phase. To consider the effect of solvent selection, initial studies investigated the preparation of 4 liposome formulations based on PC, DMPC. DPPC or DSPC mixed with equimolar cholesterol (2:1 mass ratio). Results in Figure 2 demonstrate that solvent selection plays an important role in the size of the formed vesicles; the combination of using methanol for the lipid solvent and PBS as the aqueous phase produces liposomes in the smallest size range, irrespective of the phospholipid used, with liposomes being approximately 70 to 100 nm in size. Replacement of methanol with ethanol in combination with PBS made no notable difference to liposome size in the case of PC, DMPC or DPPC liposomes. However, in the case of the DSPC:chol liposomes, preparing these vesicles using PBS and ethanol as the initial solvents results in significantly ($p<0.05$) larger vesicles that were well over 1000 nm in size. Indeed, the DSPC formulation in general tended to be more sensitive to the initial solvent selection, as switching from PBS to Tris buffer in combination with methanol also increased

the size of the DSPC:chol liposomes from 69 ± 3 nm to over 405 ± 63 nm (Figure 2). In general, the polydispersity of the liposome formulation (as measured by the PDI) followed a similar trend to the vesicle size, with the combination of methanol with PBS giving the most homogeneous preparations.

The initial lipid amount in the solvent stream is also an important consideration in the production of liposomes using microfluidics, with lower levels of lipids tending to promote larger vesicles, as shown in Figure 3. However, working with initial amounts of lipid above 1 mg (3 mg/mL) produced vesicles around 100 nm or less with good lipid recovery (Figure 3) and, irrespective of the lipid concentrations, varying the flow rate had no significant impact on vesicle size (results not shown).

3.2 Selection of flow rate and solvent to aqueous flow rate ratio

Upon selection of the two base solvents (methanol with PBS), the next stage in the process was to identify the effect of both the aqueous: solvent media (PBS:methanol) mixing ratio and also the total flow rate; therefore, the flow rate ratio was varied from 1:1 through to 5:1 and the total flow rate was varied from 5 to 15 mL per minute and the effect on the liposomal attributes (size, PDI and zeta potential) were investigated. Given that these liposomes were to be loaded with both an aqueous soluble drug (metformin) and a bilayer loaded drug (glipizide), DSPC was selected as the phospholipid based on previous studies that demonstrated longer chain lipids offer greater capacity to load drug within the bilayer of vesicles (Mohammed et al., 2004; Ali et al., 2010; Ali et al., 2013) and retain drug within the aqueous core (Gregoriadis and Davis, 1979). The cholesterol content was also reduced given that a range of previous studies have shown that cholesterol is known to reduce aqueous soluble drug leakage across the lipid bilayer (e.g. (Briuglia et al., 2015)), but also to potentially inhibit drug incorporation in the liposomal bilayer (Ali et al., 2010; Mohammed et al., 2004). Therefore, a DSPC: chol lipid weight ratio of 10:4 was selected to meet the needs of both good aqueous drug retention and bilayer drug loading.

Results in Figure 4 demonstrate that a low aqueous:solvent ratio of 1:1 tended to produce the largest vesicles, irrespective of the total flow rate, with liposomes being approximately

200 to 300 nm in size with PDI values of between 0.38 to 0.67. However, an increase in the aqueous to solvent ratio to 3:1 reduced the vesicle sizes to approximately 120 – 130 nm, and a further increase in the ratio to 5:1 reduced the vesicle size range to 80 to 90 nm with a PDI range of 0.11 to 0.22, again with no notable effect of total flow rate being seen (Figure 4). In all cases, the liposome formulations were near neutral in zeta potential as would be expected for such formulations. From these studies, it can be seen that, across the range tested, the flow rate ratio but not total flow rate had an impact on vesicle size; therefore, further studies adopted a solvent to aqueous ratio of 5:1.

3.3 Incorporation of aqueous and bilayer drug loading within liposomes manufactured by microfluidics

To investigate drug loading within both the aqueous and bilayer phases of liposomes, metformin and glipizide were selected as model drugs, given their contrasting solubility and their combined use in treating type-2 diabetes. In terms of initial drug added, 300 µg of glipizide dissolved in methanol (the maximal amount soluble in the solvent phase used; 0.27 mL) along with the DSPC and cholesterol (2.7 mg and 1.1 mg, respectively), and 20 mg of metformin was added to the PBS phase. Results in Figure 5 show that drug loading of glipizide within the liposomal bilayer was approximately 40% and metformin entrapment was approximately 20%. Furthermore, the results show that loading of the drug individually or in combination had no significant impact on the loading capacity of the liposomes. However, the presence of either drug in the formulation tended to push the vesicle size down by approximately 20 nm, with the measured z-average particle size being 50 to 60 nm. The bilayer vesicle constructs with and without the addition of these drugs as imaged by cryo-TEM are shown in Figure 6; in all 4 cases, the formulations show a high proportion of small unilamellar vesicles around 60 to 80 nm in size.

The effect of drug co-loading within the liposomal system on their relative release profiles was also investigated (Figure 7). The majority of the *in-vitro* drug release studies are based upon dialysis of liposomal formulation against large volumes of buffers or other simulated media at physiological temperatures, and this excess buffer can lead to un-realistic gradients across the liposomal membranes. Therefore, drug release was studied using USP-4 (flow

through cell (FTC) method), which has proved to be very versatile, with advantages of operating ρH gradients, achieving sink conditions for sparingly soluble drugs by use of unlimited media volume and, most importantly for the current study, appropriateness for micro-sized dosage forms, as it obviates sample aggregation. FTC enables dissolution conditions to be achieved that are more representative of physiological conditions by choosing the right flow rates, media type, media volume and cell preparation. Furthermore, FTC is the most discriminating between formulation variants (Qureshi, S.A., 2006), whilst in the closed loop system configuration, the dissolution media is re-circulated through the sample contained in a flow through cell, producing a cumulative curve progression, with the added advantage of a small volume in which dissolution takes place; the 22.6 mm-cell without glass beads provides 19 mL capacity (Brown, W. 2005). The results show that, in both cases, the drug incorporated individually had a slower release rate profile compared to the liposomes containing both metformin and glipizide. For example, after 1 hour, liposomes containing glipizide released 3% of the bilayer loaded glipizide; however, when metformin was also present within the liposome formulation, glipizide release increased to 12%. Similarly, after 1 hour metformin release increased from 35% to 64% when glipizide was present in the bilayer (Figure 7), suggesting that co-loading of both drugs on the system had an impact on the structural attributes of the liposomes.

4. Discussion

The use of microfluidics for the scalable production of liposomes allows for the cost-effective and rapid production of liposomes. Despite increased research in exploring different microfluidics parameters on liposome size, the effect of the organic solvent (used to dissolve lipids) on liposome size has not been fully explored. During this microfluidics-based manufacturing process, it has been proposed that the liposomes form as a result of the alcohol and aqueous buffer mixing, thereby increasing the polarity of the solvent. This in turn progressively decreases the lipid solubility, thereby promoting self-assembly into planar lipid bilayers. As these planar bilayer discs grow, the surface area of hydrophobic chains exposed to polar solvent around the perimeter of the disc will grow and increase the interfacial tension. To circumvent this, the discs will bend and eventually close into spherical vesicles.

Most commonly, isopropyl alcohol has been used to dissolve lipids, with some studies using the less toxic solvent, ethanol (Carugo et al., 2016). As shown in figure 2, despite all four lipids being soluble in both methanol and ethanol, the organic solvent of choice affects the liposome size attributes, potentially due to the differences in the self-assembly rates/configurations of the discs and resultant vesicles. Previous investigations into use of microfluidics to prepare liposomes (Zook and Vreeland, 2010) have shown that vesicle size is modulated by temperature in the case of high-transition temperature lipids. The authors note this to be due to the high membrane elasticity modulus associated with high transition temperature lipids, with liposomes formed below or near the transition temperature of the lipids tending to be larger. In contrast, liposomes formed at a temperature far above the transition temperature have a much smaller dependence of size, where the membrane elasticity modulus is relatively constant (Zook and Vreeland, 2010). Therefore, whilst cholesterol is present in the formulations and could nullify the impact of the DSPC transition temperature, during nanoprecipitation and formation of liposomes, these transition temperatures may have an impact.

An additional issue with the use of ethanol may be that the residual presence of ethanol in the liposome suspensions may also promote vesicle fusion; previous studies have shown that increased ethanol concentration causes aggregation of liposomes produced by microfluidics at a 1:1 FRR, and increasing the FRR to 3:1 removed the aggregation problem and formed smaller liposomes (Maeki et al., 2015). Furthermore, the increased vesicle size may be a result of the residual ethanol that may accumulate at the organic and aqueous interface (Patra et al., 2006) thereby promoting vesicle fusion. Using computational studies to explore the effect of methanol and ethanol on the DPPC lipid bilayer, Patra et al (2006) showed that ethanol affects the structural properties of liposome bilayers, with this effect possibly more prominent with the DSPC formulation due to the longer alkyl chains.

The results in Figure 4 show that manipulating flow conditions such as the speed at which both streams, aqueous and organic, pass through the channels (TFR) did not affect the size of the liposomes. On the other hand, the ratio between aqueous and organic phases (FRR) has shown to be a key parameter in the control of the liposome size. This could be explained due to the increase of aqueous volume that favours the formation of liposomes, since the lipid in solvent is diluted (Jahn et al., 2004; Zook and Vreeland, 2010). Therefore, increasing FRR

produces a dilution effect, reducing amount of solvent (methanol in our case), and a faster mixing. By this means the formation of larger liposomes by particle fusion and lipid exchange is reduced (Zhigaltsev et al., 2012). In contrast, at low FRRs, the organic solvent is injected into the system more slowly, allowing more time for both streams to interact, and therefore, producing larger liposomes (Zhigaltsev et al., 2012). These results show that control of the flow rate ratio can control vesicle size and that the rate of liposome production (in terms of total flow rate) may be increased without impact on the liposome attributes.

In terms of drug loading within these vesicles prepared using microfluidics, the loading was based on the principle of passive loading, where both drug and lipids are co-dispersed in the aqueous phase. Generally, encapsulation efficiency for passive loading is less than 10% (Cullis et al., 1989), whilst within our studies, we achieve notably higher hydrophilic (metformin) drug loading of approximately 20% (Figure 5). The use of microfluidics has been suggested to improve hydrophilic loading; For example, Jahn et al., 2008 reported unexpectedly high entrapment efficiencies of a hydrophilic moiety (sulforhodamine B dissolved in PBS) within nanometer-scale liposomes prepared using a continuous-flow microfluidics system. The authors suggest that the high encapsulation efficiency may be due to a spatial concentration enhancement induced by viscosity anisotropy in the microchannel (Jahn et al., 2008). In terms of bilayer loading, the simultaneous packaging of the lipids and glipizide within the bilayer can promote drug loading of approximately 40%, similar to our previous studies with propofol (Kastner et al., 2015). The small decrease in size noted when liposomes were formed in the presence of metformin and/or glipizide may be a result of changes in viscosity, miscibility and/or mixing at the interphase as the liposomes form as discussed by Jan et al. (2008).

In terms of drug release, when both types of drugs are co-entrapped, our studies demonstrated that they release faster than when individually encapsulated, suggesting an interference or synergistic effect occurs. We hypothesised that the presence of glipizide within the liposomal bilayer may interfere with the packing density of the lipids in the small and highly curved bilayers and thereby increasing bilayer drug permeability. Furthermore, the presence of metformin within the liposomes may also induce a concentration gradient across the membrane, further driving the disruption of the bilayer, thereby simultaneously increasing the release of both drugs.

5. Conclusions

Our results provide a concise analysis of liposome manufacturing using microfluidics. Furthermore, for the first time we demonstrate the simultaneous entrapment of a hydrophilic and a lipophilic drug (metformin and glipizide) using a scalable microfluidics system. Our results demonstrate that microfluidics promotes greater hydrophilic and hydrophilic drug loading compared to traditional methods, whilst critical factors to consider in the manufacture of liposomes using microfluidics include the choice of solvent, lipid concentration, and the flow rate ratio adopted during the microfluidics process. Therefore, it can be concluded that microfluidics is a good alternative for liposome manufacturing.

6. Acknowledgements

This work was part funded by Aston University (SJ), the EPSRC Centre for Innovative Manufacturing in emergent macromolecular therapies (EP/L015218/1) (MH) and EP/I033270/1 (EK) and EU Horizon 2020 project TBVAC 2020 (Grant no. 643381) (CBR).

Supporting information Available: Data presented in this publication can be found at [DOI XXXXXXXXXXX to be provided at proofing stage].

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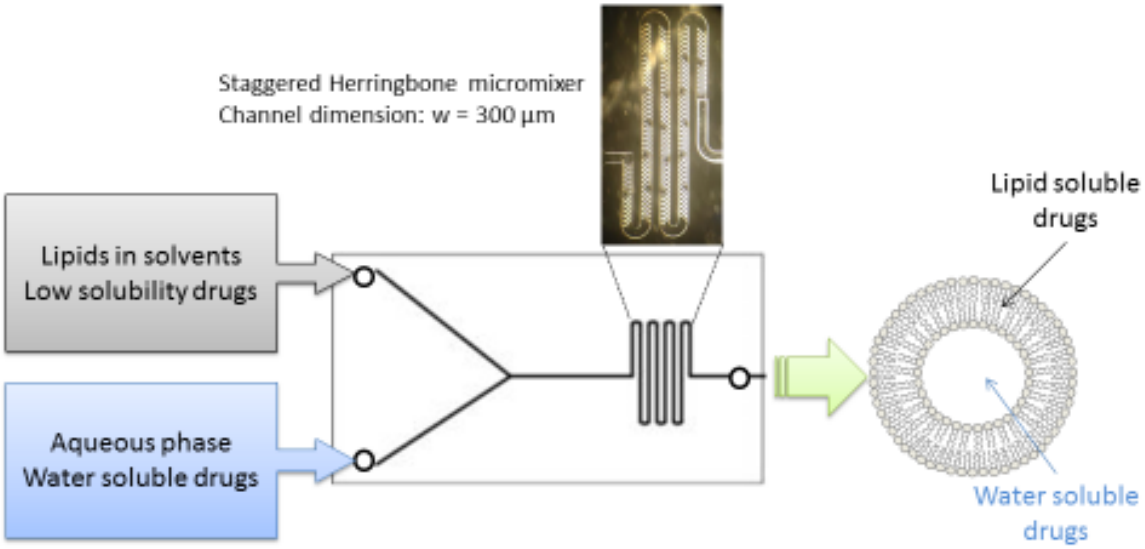
Figure legends



Input considerations	Manufacturing parameters	Critical product attributes
Solvent selection	Chip design	Vesicle size, PDI, zeta potential
Lipid and drug solubility	Total Flow rate	Drug loading and recovery
Lipid and drug concentration	Flow rate ratio	Lipid recovery
	Lipid transition temperatures	

Figure 1

Figure 1: Liposomes produced by microfluidics – process and formulation conditions. In the production of liposomes using microfluidics, the lipids and lipophilic drugs are dissolved in an appropriate solvent and mixed with an aqueous phase containing water soluble drugs. These are mixed using micromixers that are available in a range of designs. Within these studies, a staggered herringbone micromixer was used. The rate of mixing of the aqueous and solvent buffer and the total follow rate are also parameters that require optimisation and the lipid concentration and lipid transition temperature may impact on this optimisation process. In terms of critical product attributes, key factors to consider include the liposome physico-chemical attributes (including size, pdi, zeta potential), drug loading and drug release profiles and lipid recovery.

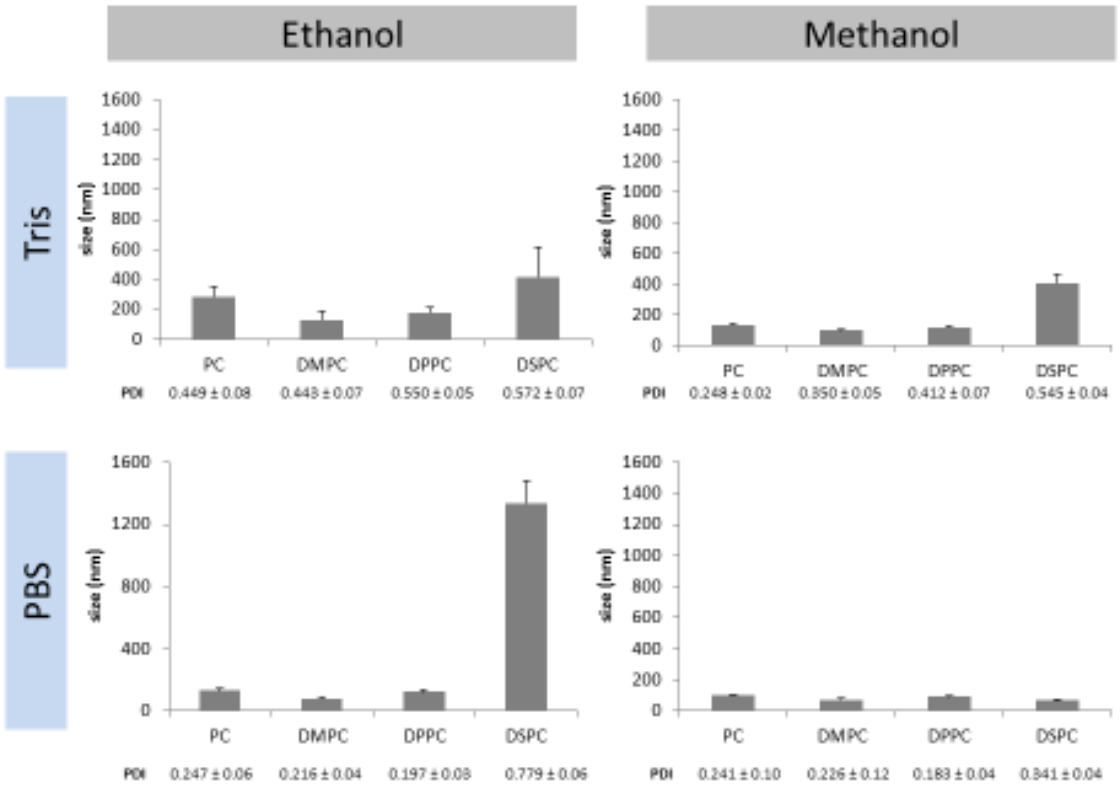


Figure 2:

Figure 2: The effect of solvent and buffer selection on liposomes prepared by microfluidics. Liposomes composed of PC, DMPC, DPPC, DSPC and cholesterol (1:1 molar ratio/2:1 mass ratio) were prepared by microfluidics at a 3:1 Flow rate ratio and 15 mL/ min Total Flow rate. Lipids were either dissolved in ethanol or methanol. Tris buffer or PBS were used as the aqueous phase, with size and PDI measured. Results are expressed as the means of three independent experiments ± SD.

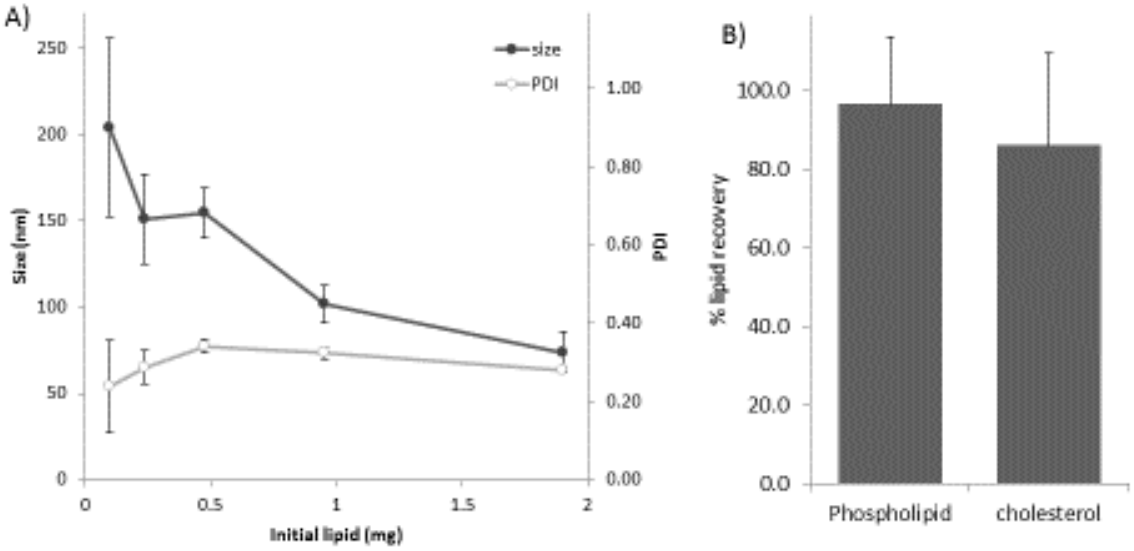
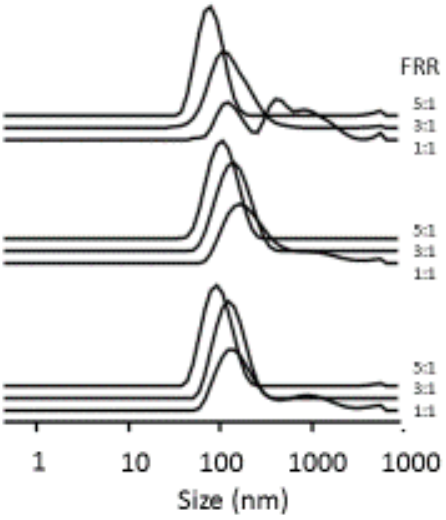


Figure 3.

Figure 3: Consideration of A) initial lipid amount and B) lipid recovery. Liposomes composed of PC and cholesterol (1:1 molar ratio/2:1 mass ratio) were prepared by microfluidics at a 3:1 flow rate ratio using increasing amounts of initial total lipid, with size with size and PDI measured. Results are expressed as the means of three independent experiments ± SD. For lipid recovery, results are averaged across a range of PC, DMPC, DPPC and DSPC in equimolar concentration with cholesterol. Results are expressed as the means of at least three independent experiments ± SD.

Flow Rate	Solvent to aqueous ratio	Z-average diameter (nm)	PDI	Zeta potential (mV)
5 mL/min	1:5	91 ± 4	0.153 ± 0.03	-9.4 ± 2
	1:3	121 ± 7	0.133 ± 0.02	-4.4 ± 2
	1:1	181 ± 13	0.380 ± 0.15	-2.8 ± 1
10 mL/min	1:5	100 ± 5	0.109 ± 0.04	-4.0 ± 2
	1:3	129 ± 9	0.130 ± 0.05	-2.2 ± 1
	1:1	209 ± 21	0.348 ± 0.13	-0.1 ± 1
15 mL/min	1:5	81 ± 6	0.224 ± 0.04	-10 ± 3
	1:3	121 ± 8	0.229 ± 0.05	-5.0 ± 1
	1:1	308 ± 17	0.667 ± 0.09	-1.0 ± 1



538 Figure 4.

539 Figure 4: The effect on flow rate ratio and total flow rate on the liposomes z-average
540 diameter, PDI and zeta potential of DSPC:cholesterol liposomes. Results represent mean ±
541 SD, n = 4.

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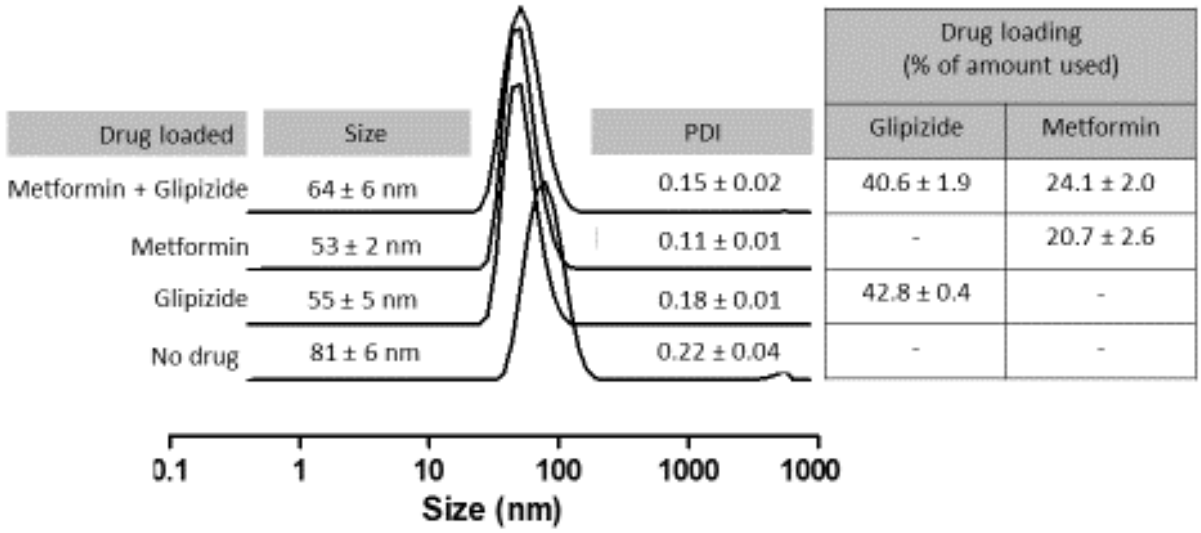

543 Figure 5

544 Figure 5: A comparison of DSPC:cholesterol liposomes z-average diameter, PDI and drug
545 loading for small unilamellar liposomes with glipizide loading within the bilayer, with
546 metformin loading within the aqueous phase, liposomes containing both glipizide and
547 metformin and liposomes without drug present. Results represent mean ± SD, n = 4.

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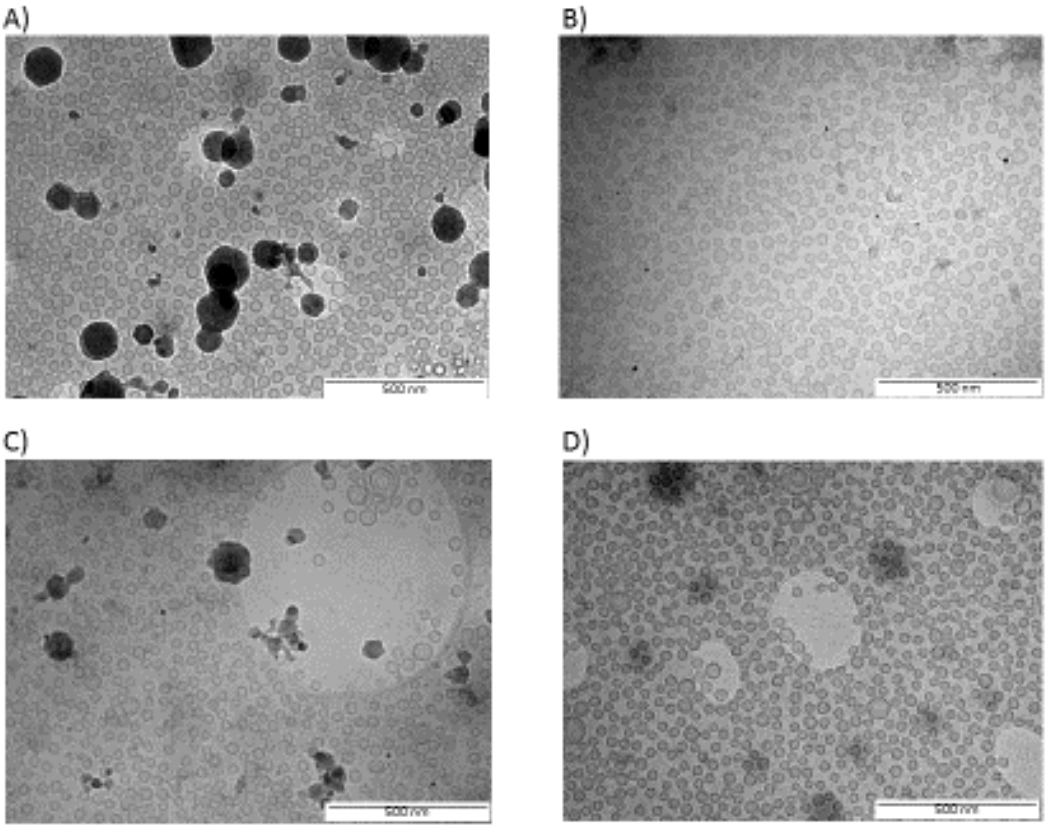

549 Figure 6

550 Figure 6: DSPC:Cholesterol liposomes produced by microfluidics at a 5:1 flow rate ratio and
551 15 mL/min and imaged using cryo-TEM. A) Liposomes without drug incorporated, B)
552 liposomes with glipizide loading within the bilayer, C) liposomes with metformin loading
553 within the aqueous phase D) liposomes containing both glipizide and metformin. The size bar
554 represents 500 nm.

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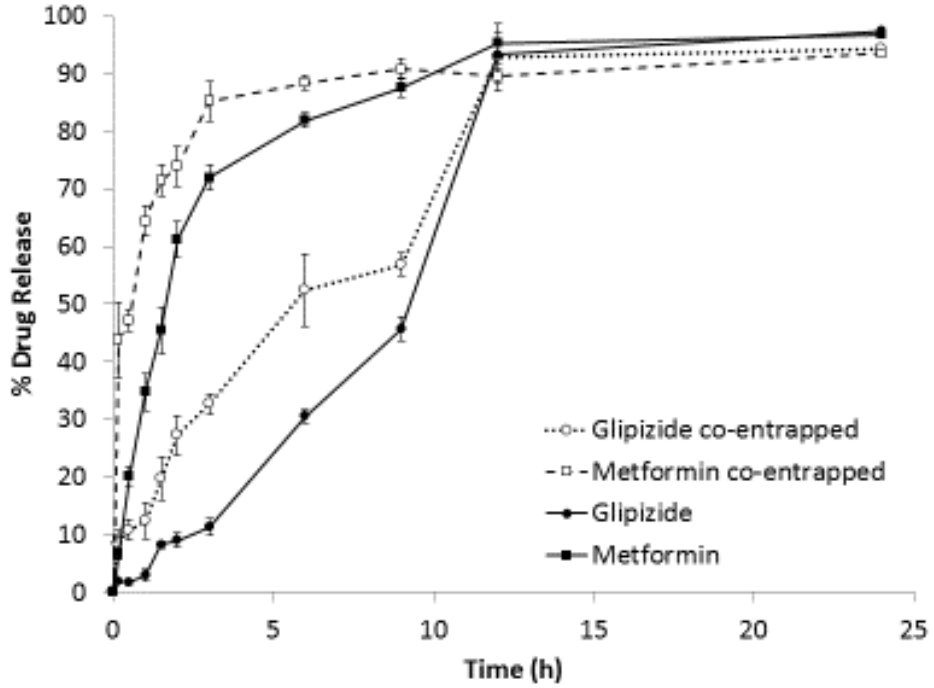

Figure 7.

Figure 7: Drug release profiles, measured using USP IV, from liposomes incorporating glipizide or metformin individually, or co-encapsulated within DSPC:chol liposome formulations produced via microfluidics as outlined in Figure 5. Release studies were undertaken with PBS (pH=7.4), temperature 37 ^{o}C and drug concentrations quantified by RP-HPLC. Results represent mean ± SD, n = 4.