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Title: Rapid scale-up and production of active-loaded PEGylated liposomes

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Abstract

Manufacturing of liposomal nanomedicines (e.g. Doxil®/Caelyx®) is a challenging and slow process based on multiple-vessel and batch processing techniques. As a result, the translation of these nanomedicines from bench to bedside has been limited. Microfluidic-based manufacturing offers the opportunity to address this issue, and de-risk the wider adoption of nanomedicines. Here we demonstrate the applicability of microfluidics for continuous manufacturing of PEGylated liposomes encapsulating ammonium sulfate (250 mM). Doxorubicin was subsequently active-loaded into these pre-formed liposomes. Critical process parameters and material considerations demonstrated to influence the liposomal product attributes included solvent selection and lipid concentration, flow rate ratio, and temperature and duration used for drug loading. However, the total flow rate did not affect the liposome product characteristics, allowing high production speeds to be adopted. The final liposomal product comprised of 80 -100 nm vesicles (PDI <0.2) encapsulating ≥ 90% doxorubicin, with matching release profiles to the innovator product and is stable for at least 6 months. Additionally, vincristine and acridine orange were active-loaded into these PEGylated liposomes (≥ 90% and ˜100 nm in size) using the same process. These results demonstrate the ability to produce active-loaded PEGylated liposomes with high encapsulation efficiencies and particle sizes which support tumour targeting.

Key words: microfluidics, high-throughput, scalable, cost-effective, PEGylated liposomes, doxorubicin, vincristine, acridine orange.

1. Introduction

The manufacture of nanomedicines presents challenges to the pharmaceutical sector, often limiting their translation from bench to bedside. This is due to the limited feasibility of scaling most conventional bench-scale techniques to clinical scale production. Indeed, despite their recognised benefits for drug delivery, few (<50) liposomal products have reached the market (Sercombe et al., 2015). Amongst these, Doxil®/Caelyx® (US/UK), was the first nanomedicine approved by the Food and Drug Administration (FDA) in 1995. Doxil®/Caelyx® is a PEGylated liposomal formulation composed of three lipids: fully hydrogenated phosphatidylcholine (HSPC), cholesterol (Chol) and *N*-(carbonyl-methoxyPEG 2000)-1,2-distearoyl-sn-glycero-3-phosphoethanolamine sodium salt (DSPE-PEG2000) at 16 mg/mL total lipid concentration (3:1:1 w/w respectively). This lipid combination provides a rigid liposome bilayer at physiological temperature (37°C) promoting drug retention (Gabizon et al., 2003; Wei et al., 2016). The small vesicle size (80 to 100 nm), and the MPEG moiety (which extends into the aqueous phases (Rafiyath et al., 2012)) increase the liposome *in vivo* half-life by reducing opsonisation and clearance by the mononuclear phagocytic system. The liposome suspension contains 2 mg/mL of the anthracycline doxorubicin HCl, mainly encapsulated in the aqueous core as doxorubicin-sulfate crystals via an ammonium sulfate ($[NH_4]_2SO_4$) gradient between the interior and exterior of the liposomes. The liposome suspension is stored at 5°C in histidine buffer containing sucrose for isotonicity (10 mM histidine, 10% w/v sucrose pH 6.5), and it is administered intravenously to treat a range of cancers including metastatic breast cancer, advanced ovarian cancer, multiple myeloma and Kaposi's sarcoma (Gabizon et al., 2003). By incorporation of doxorubicin within PEGylated liposomes, targeting to the tumour sites is increased up to 10-fold due to the enhanced permeation and retention (EPR) effect, thus reducing off-site toxicity (Akbarzadeh et al., 2013; Wong et al., 2015). Once the PEGylated liposomal doxorubicin reaches the tumour site, the drug is preferentially released in the tumour cells due to the acidic pH encountered there (Silverman and Barenholz, 2015; Wong et al., 2015). Through this targeting, reduced cardiomyopathy, nausea, alopecia and vomiting have been observed (Rafiyath et al., 2012). However, PEGylated liposomes do promote adverse events caused by the accumulation of PEG in the skin and the appearance of palmar–plantar erythrodysesthesia (hand–foot syndrome) (Akbarzadeh et al., 2013; Jiang et al., 2011).

Despite the success of Doxil®/Caelyx® (current market value forecast to be approximately 300 Million USD by 2024 (GrandViewResearch, 2016)) liposome and nanoparticle development in general faces numerous challenges including scale-up practicability, reproducibility and complex regulatory aspects. Thus, liposome production can be arduous to manufacture. Indeed, in 2011, the FDA raised an injunction against the production company due to the failure to comply with Good Manufacturing Practice (GMP) requirements in relation to the production of Doxil®/Caelyx® (EMA, 2011).

Subsequently, the FDA approved a generic liposomal doxorubicin (Lipodox®) to address supply shortages (FDA, 2013). However, production of this generic product is still a multi-step batch, time-consuming method, which consist of at least a 5-day batch production (Palmer, 2013). Therefore, to improve accessibility and exploitation of liposomes and nanomedicines, a robust and scalable manufacturing process is needed. To address this, new microfluidic processes are being used (Dong et al., 2019; Forbes et al., 2019; Roces et al., 2020; Roces et al., 2019; Shah et al., 2019; Wagner et al., 2002; Webb et al., 2019). The advantages of adopting microfluidics as part of the production process, includes robust particle size control and high reproducibility across production scales and hence the ability to support scale-independent and/or continuous operation. It is easy to control, automated and also cost-effective. For example, microfluidics has been used to prepare drug-loaded PEGylated liposomes in a range of sizes such that the impact of liposome size on tumour retention and penetration could be investigated (Dong et al., 2019). The authors demonstrate that the use of microfluidics allows smaller vesicles to be prepared compared to sonication, and that smaller liposomes (~50 nm) promoted deeper tissue penetration compared to larger (~75 nm) liposomes (Dong et al., 2019).

Therefore, building on these previous studies, we have developed a scale-independent method for the production of liposomal doxorubicin using microfluidics. We have identified and optimised the critical process parameters during this manufacturing method and their linked impact on the critical quality attributes (CQA) of the final product. The main parameters affecting the microfluidic liposome production are the flow rate ratio, the total flow rate and heating block. Flow rate ratio is the ratio between the aqueous and the organic phase, and it has been reported several times (e.g. Forbes et al., 2019; Jahn et al., 2010; Zizzari et al., 2017) to impact the liposomal size due to the differences in polarity between both phases. Total flow rate is the speed at which both phases run through the micromixer. This parameter has shown little or no impact on the characteristics of the produced liposomes in the majority of the published papers, however, few researchers have reported its impact during manufacturing (e.g. Maeki et al., 2017; Roces et al., 2019). The effect of the total flow rate could hamper the production process due to increased manufacturing times. The use of a heating block might be needed for the production of liposomes containing high transition temperature lipids (e.g. Roces et al., 2019). However, this would add an extra parameter to control during manufacture, thus, avoiding the use of temperature would simplify the production process (e.g. Forbes et al., 2019). For active loading of drugs, the time and temperature applied during the loading process will affect the amount of drug encapsulated (Zucker et al., 2009), which will contribute to the toxicity or efficacy of the formulation. In terms of material preparation, the selection of the aqueous phase (e.g.(Lou et al., 2019)), organic phase (e.g.(Webb et al., 2019)) and lipid concentration (e.g. Forbes et al., 2019)

can all influence the CQA of the produced liposomal product. Thus, evaluation and optimisation of these process parameters is key when manufacturing liposomal products using microfluidics.

In the case of Doxil®/Caelyx®, specific characteristics have been reported critical to demonstrate the quality and consistency of the final product. These reported CQA consist of liposome size, size distribution, lamellarity, zeta potential, lipid composition, encapsulation efficiency, state of the encapsulated drug and *in vitro* drug release profiles (Jiang et al., 2011). Some of these properties (e.g. particle size and/or surface charge) are commonly related to the *in vivo* pharmacokinetics and pharmacodynamics of the product and their efficacy (Di Francesco et al., 2017; Jiang et al., 2011). Thus, we have characterised PEGylated liposomal doxorubicin manufactured using a microfluidic production method which incorporates tangential flow filtration for the scalable manufacture. This study assessed the process parameters and their impact on the CQA of the final product. The produced formulations were compared to the marketed product (Caelyx®/Doxil®). Additionally, vincristine and acridine orange encapsulation into PEGylated liposomes was assessed to consider other actively loaded drugs and the possibility of 'just-in-time drug loading'. Building on initial work demonstrating the formulation of doxorubicin-loaded PEGylated liposomes using this commercial microfluidic technology (e.g. (Cheung and Al-Jamal, 2019; Dong et al., 2019)), here we demonstrate a comprehensive evaluation of the key process parameters and probe the ability of this process to de-risk the translation of liposome production from bench to clinic scale.

2. Materials and Methods

2.1 Materials

Hydrogenated soy L-α-phosphatidylcholine (HSPC, >99%), 1,2-distearoyl-sn-glycero-3-phosphocholine (DSPC, >99%), soy phosphatidylcholine (Soy PC, >99%) and 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy(polyethylene glycol)-2000] (ammonium salt) (DSPE-PEG2000, >99%) were purchased from Lipoid (Ludwigshafen, Germany). Cholesterol (chol, >99%), doxorubicin hydrochloride (DOX) European Pharmacopoeia Reference Standard, acridine orange (75% dye content), vincristine sulfate (95 – 105%), sodium azide (NaN_3, >99%), ammonium bicarbonate (NH_4HCO_3, >99%), 2-(N-morpholino) ethane sulfonic acid (MES, >99%), 5% w/v hydroxypropyl-cyclodextrin (HP-CD), 1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate (DilC, >98%), histidine >99%, sucrose >99% and ammonium sulfate ($[NH_4]_2SO_4$, >99%) were obtained from Sigma-Aldrich Ltd. (Poole, UK). Phosphate buffered saline tablets (10 mM, PBS pH 7.3) were acquired from Oxoid Ltd (Basingstoke, UK). All other reagents (ethanol, methanol, isopropyl alcohol) were of

analytical grade (HPLC grade, >99.8%) and purchased from commercially available suppliers. Ultrapure-water was provided by an in-house system.

2.2 Manufacture of PEGylated liposomes encapsulating ammonium sulfate

PEGylated liposomes were prepared using the microfluidic platform from Precision NanoSystems Inc. (Vancouver, Canada). Optimisation of the formulations was performed on the bench scale system (Nanoassemblr™ Benchtop) which uses a staggered herringbone micromixer (SHM). Liposomal formulations containing either HSPC or DSPC (20 - 40 mg/mL stock), cholesterol (8 mg/mL stock) and DSPE-PEG2000 (10 mg/mL stock) at a w/w ratio of 3:1:1 respectively (56:39:5 molar ratio) were produced. When high concentrations were used (e.g. 40 mg/mL total lipid), the lipid combination was mixed together in one stock and then solubilised in ethanol. Lipids were heated up for solubilisation, but once in solution the mixture was stable. Lipids were allowed to reach room temperature before injection into the microfluidics system. Ammonium sulfate ($[NH_4]_2SO_4$) 250 mM was used as aqueous phase. The effect of the formulation parameters such as lipid choice (HSPC vs DSPC), solvent effect (ethanol, methanol or a mixture of both) and initial lipid concentration (from 2.5 to 40 mg/mL) were tested. The effect of microfluidic process parameters, flow rate ratio (FRR), total flow rate (TFR) and temperature were also evaluated. Therefore, DSPC/HSPC:Chol:DSPE-PEG2000 liposomes were produced encapsulating $[NH_4]_2SO_4$ 250 mM. To test the ability of the release study methods to discriminate between formulations, SoyPC:Chol:DSPE-PEG2000 and HSPC:LowChol:DSPE-PEG2000 formulations (low cholesterol formulation, containing 25 w% less than the original) were also prepared.

2.3 Purification and buffer exchange of PEGylated liposomes using tangential flow filtration

Once the PEGylated liposomal formulations encapsulating $[NH_4]_2SO_4$ 250 mM were produced, the next step was to remove the solvent while simultaneously conducting buffer exchange to establish a pH gradient between the liposome interior and the external medium for the active loading of drugs. For this purpose, a tangential flow filtration system (KR2*i* TFF System®, Repligen, Waltham, US) using a 500 kDa modified polyethersulfone (mPES) column and masterflex tubing 13 was used. 20 mL/min flow rate and 12 diafiltration volumes with either PBS (pH 7.3, 10 mM) or Histidine-Sucrose (pH 6.5, 10 mM-10% w/v) were applied. Unless otherwise stated, samples were recovered to the initial volume. This step resulted in PEGylated liposomes encapsulating acidic pH ($[NH_4]_2SO_4$, pH 5.5) and suspended in physiological pH (PBS pH 7.3 or His-Suc pH 6.5), creating a gradient for the diffusion of drugs with protonisable amine functions.

A concentration step was included for the preparation of some formulations. In this case, formulations prepared at 4 mg/mL were concentrated 4-fold to 16 mg/mL using tangential flow filtration. To avoid damage of the tangential flow filtration column membrane and accelerate the purification process by reducing the pressure within the column, liposomes were diluted 2-fold with $[NH_4]_2SO_4$ 250 mM to reduce the ethanol content from 40% (FRR 1.5:1) to 20% volume.

A continuous tangential flow filtration system was developed for larger volumes (>50 mL). Product was filtered through a custom built tangential flow filtration system, which consisted of 3 in-line hollow fibre filters. The product was driven through the filter using a peristaltic pump at 10 mL/min. The retentate outlet was restricted to 5 mL/min to concentrate the product, with subsequent dilution of the retentate using 10 mM phosphate buffered saline at 5 mL/min. The product was concentrated and diluted through the 2nd and 3rd filter to give a filtered and buffer exchanged product.

2.4 Head-space gas chromatography

Quantification of the residual solvent within the product was performed using a head-space gas chromatography Clarus 580 system with a flame ionisation detector (Perkin Elmer, Beaconsfield, UK) with a 30 m x 0.32 mm x 0.25 ID column (Elite Wax – S/N 1627777). For sample preparation, 1 mL internal standard (n-propanol, 1% v/v in de-ionised water) was added to 2 mL of standard/sample. This was subsequently capped and placed in an oven at 65°C for 20 min, alongside the head-space syringe. Once removed from the oven, 10 µL were sampled and analysed using the head-space system. The instrument method was set as follows: 35°C (for 2 minutes), ramp at 10°C/min to 100°C and held at 100°C for 0.5 minutes. Inlet temperature was set at 100°C, the detector temperature 250°C, 45 mL/min H_2 and 450 mL/min air. Helium (1 mL/min, split ratio 1:3) was used as a carrier gas.

2.5 Active-loading of drugs into PEGylated liposomes

Doxorubicin was added into the pre-formed liposomes via active loading. Briefly, liposomes were produced using microfluidics at 4 mg/mL (final concentration) and purified using tangential flow filtration. Doxorubicin stock was prepared at 10 – 30 mg/mL in ultrapure water. A specific volume was then added into the washed liposomes at 0.125 g doxorubicin/g lipid. Subsequently, the effect of the loading time and loading temperature, on the liposomal formulation containing doxorubicin was evaluated. Formulations were incubated in a water bath at different temperatures (20°C, 40°C and 60°C) and for a specific time (generally, until 90% encapsulation efficiency (EE) was reached). Furthermore, the effect of the external buffer used (PBS vs His-Suc) was also compared. After loading,

formulations were cooled down to room temperature, and non-encapsulated doxorubicin was removed using tangential flow filtration again. For the optimisation of the tangential flow filtration cycle, free doxorubicin at 0.25, 0.5 and 2 mg/mL was used, and the amount of doxorubicin in the filtrates was quantified.

Quantification of doxorubicin was performed using a microplate reader model 680 (Bio-rad Laboratories. Inc., Hertfordshire, UK) measuring the UV absorbance at 490 nm. Liposomal samples were mixed with 50% isopropyl alcohol in order to disrupt the liposome membrane and quantify the entire drug content. Calibration curves were performed under the same conditions as the samples. EE% was calculated as the percentage of doxorubicin quantified divided by the theoretical amount added into the formulation.

Vincristine and acridine orange were also encapsulated within the optimised HSPC:Chol:DSPE-PEG2000 formulation. These drugs were incubated at 60°C for 30 min. The drug-to-lipid ratio and absorbance used were 0.071 and 0.004 g drug/g lipid, and 295 and 400 nm for vincristine and acridine orange respectively.

2.6 *In vitro* drug release profiles

Several types of release studies were performed to assess the doxorubicin release profiles and to evaluate if they were able to discriminate between formulations prepared with different lipid composition, particle size or loading conditions. In all three tests, 1 mL aliquots were withdrawn at each time point, and same volume used to replenish (using the assay media) to maintain sink conditions. Vessels were protected from the light due to the photosensitivity of doxorubicin.

2.6.1 USP-4 dissolution release studies

Doxorubicin release was carried out following the optimised method from Yuan et al. using a USP-4 apparatus (SOTAX®) assay in a closed loop at 16 mL/min (Yuan et al., 2017). The release media was freshly prepared and consisted of 100 mM NH_4HCO_3, 75 mM 2-(N-morpholino) ethane sulfonic acid (MES), 5% w/v hydroxypropyl-cyclodextrin (HP-CD) to avoid doxorubicin precipitation, 5% w/v sucrose and 0.02% w/v NaN_3 (pH 6). The drug release assay was performed at 45°C in a closed loop. Aliquots were taken at 0.5, 1, 2, 3, 4, 6 and 24 h. A dialysis membrane with a cut-off 300 kDa (Repligen, Waltham, US) was used. The media volume per cell was 39.2 mL, and the sample volume 0.8 mL (16 mg/mL total lipid concentration encapsulating 2 mg/mL doxorubicin). Free drug (2 mg/mL) was used as a control on each run.

The cumulative release was calculated (as described by Yuan et al. (Yuan et al., 2017)) as the percentage of the calculated doxorubicin concentration from the liposomes at each time point, divided by the detected concentration of free doxorubicin from the control at the same time point. A UV plate reader at 490 nm was used for this purpose (300 µL/well).

2.6.2 Dialysis: Reverse gradient and simulated in vivo testing

A reverse gradient method was developed using a 300 kDa cut-off dialysis membrane (Repligen, Waltham, US), 19.6 mL media volume ($[NH_4]_2SO_4$ pH 6.6, 250 mM) and 0.4 mL sample volume (4 mg/mL total lipid concentration encapsulating 0.5 mg/mL doxorubicin) and free drug (0.5 mg/mL as a control) at 45°C in a shaking waterbath (60 rpm). Aliquots were taken at 0, 0.5, 1, 2, 3, 4, 5, 6, 24 and 48 h time points. This study was validated in terms of limit of detection and quantification, inter- and intra-day variability and linearity (Figure S1).

Simulated *in vivo* release was performed using a different dialysis method. Liposomal samples (1 mL containing 4 mg/mL total lipid concentration and 0.5 mg/mL doxorubicin) were inserted in a dialysis tubing (cut-off 12000 Da; Sigma-Aldrich Ltd., Poole, UK) and added to a beaker filled with 20 mL release media (either PBS or PBS + 1% human serum). The waterbath was set at 37°C temperature and 60 rpm. Time points 0, 1, 2, 3, 4, 5, 6 and 8 h.

Quantification of the percentage of doxorubicin released was performed using a fluorimeter (POLARstar® Omega, BMG Labtech, Ortenberg, Germany) (excitation 501 nm; emission 550 nm; gain 1500). Therefore, 200 µL of sample was added into a black 96-well plate for the preparation of the calibration curve and the aliquots. Doxorubicin release was quantified as the percentage cumulative release. Volume sample withdrawn (mL)/ bath volume (mL)* P(t-1) + Pt, where P(t-1) is the percentage released previous to t and Pt is the percentage released at time t.

2.7 Determination of the physicochemical characteristics of the produced particles

The physicochemical characteristics of the liposomes: z-average diameter (d.nm), polydispersity and zeta potential, were measured using the Malvern nanoZS from Malvern Panalytical (Worcestershire, UK). Liposomal formulations were diluted in ultrapure water to a final concentration of 0.2 mg/mL in order to get an attenuator position between 6 - 7. Samples were equilibrated and three measurements at 25°C were conducted on each of the samples. Same dilution was used to measure the zeta potential of the particles using electrophoretic mobility. Mean particle size and PDI are expressed as the mean ± standard deviation.

Particle counting was measured using microfluidic resistive pulse sensing (MRPS; Spectradyne ncs1) which allows for individual particle counting. Liposomal samples were diluted 1:300 in PBS and measured using a T-300 cartridge.

2.8 Stability study

PEGylated liposome suspension encapsulating doxorubicin were stored at 4 ± 3°C during a period of 6 months. The stability was evaluated by measuring the physicochemical liposome characteristics particle size and particle size distribution (PDI) during storage at selected time points. Doxorubicin retention was quantified at the end of the experiment.

2.9 High throughput manufacture of PEGylated liposomes

The optimised formulation, consisting of HSPC:Chol:DSPE-PEG2000 dissolved in ethanol at an initial lipid concentration of 10 mg/mL and $[NH_4]_2SO_4$ 250 mM as aqueous phase, was prepared at 1.5:1 FRR (therefore, 4 mg/mL final lipid concentration) using the high throughput systems (scale-up) from Precision NanoSystems Inc. (Vancouver, Canada). This platform uses two types of cartridges: the SHM (bench scale and scale-up up to 20 mL/min) and the toroidal mixer (TrM, for higher speeds >20 mL/min). Therefore, HSPC:Chol:DSPE-PEG2000 liposomes produced using the scale-up system were purified and washed with PBS using tangential flow filtration, following the protocol mentioned previously. Doxorubicin was loaded during 10 min at 60°C and EE% calculated using UV at 490 nm. The liposome preparations obtained were characterised for their size, polydispersity index, zeta potential, EE% and *in vitro* leakage.

2.10 Morphological characterisation of PEGylated liposomes: Cryogenic Transmission Electron Microscopy (Cryo-TEM)

Cryo-TEM samples were prepared using a Gatan CP3 cryoplunge and imaged using a JEOL 2100 Plus operating at 200 kV. The liposomal samples (3 µL; 4 mg/mL total lipid) were deposited onto a holey carbon/300 mesh copper grid, held in tweezers within a controlled environment (~25°C, 70 - 80% humidity), and blotted (1.5 sec), before plunging to vitrify into liquid ethane (-172°C). During transfer to the TEM the sample was maintained under liquid nitrogen (-196°C) (Gatan 926 cryo sample holder), with the temperature held around –176 °C throughout imaging with a Gatan (Smartset model 900)

cold stage controller. Images were recorded (Gatan Ultrascan 100XP camera) with a nominal underfocus value of 3 - 5 µm and an objective aperture (typically 60 µm) to enhance phase contrast.

2.11 Liposome yield quantification

Quantification of the liposome yield was carried out following a protocol where a lipophilic fluorophore, DilC, is added into the lipid phase before microfluidics (Forbes et al., 2019). 0.1 mol% is added into the formulation and liposome fluorescence is measured before and after purification with tangential flow filtration. The optic settings were set at 544 - 590 nm excitation/emission and gain 1000 (POLARstar® Omega, BMG Labtech, Ortenberg, Germany). The recovery was calculated as the percentage of the initial amount using a calibration curve containing DilC-labelled liposomes after microfluidics.

2.12 Sterilisation of the PEGylated liposomes

PEGylated liposomes were sterilised using a sterile polyethersulfone (PES) 33 mm syringe filter unit 0.22 µm (Millipore, Millex-GP). Liposome yield was quantified before and after sterilisation using DilC-labelled liposomes. Drug recovery was quantified using UV at 490 nm. This procedure was assessed in empty and doxorubicin-loaded HSPC:Chol:DSPE-PEG2000 liposomes at 16 mg/mL total lipid concentration.

2.13 Statistical analysis

One-way ANOVA and two-way ANOVA followed by Tukey's multiple comparison test (p value of less than 0.05) were used for the data analysis. All the experiments were carried out at least in triplicate unless otherwise stated, and are shown as the mean of the measurements ± standard deviation which is plotted as error bars. For the statistical analysis of the drug release studies from various formulations, the f_2 similarity test was followed.

3. Results and discussion

Traditional methods for liposome production are commonly multi-step and time-consuming processes based on the rehydration of lipids using an aqueous phase for the formation of lipid vesicles varying

in size, size distribution and lamellarity. These production methods are scale-dependent and thus, for the creation of scalable liposomal production techniques, the major focus has been on single-step techniques based on the fluid control within microchannels, to produce uniform small unilamellar vesicles. The major drawback of this technique based on the fluid control is the residual solvent, the lipid concentration strength and the existence of free active pharmaceutical ingredient. However, control of these parameters is achieved with the incorporation of tangential flow filtration units. Both, microfluidic technology and tangential flow filtration are scalable processes which could include process analytical techniques to control the CQA of the product and therefore, facilitate the translation from bench to bedside medication (Worsham et al., 2019). Microfluidics has been previously compared to conventional liposomal manufacturing methods such as lipid film hydration (Roces et al., 2019) and extrusion (Shah et al., 2019), giving comparable *in vitro* and *in vivo* efficacy. Given that both particle size and polydispersity are recognised as CQA of liposomes (as they affect liposomal pharmacokinetics and pharmacodynamics (D'Mello et al., 2017)), initial studies focused on the impact of process parameters on these attributes.

3.1 Optimisation of microfluidic production parameters using the bench scale microfluidic system

3.1.1 Liposome size controlled via flow rate ratio

Initially two liposomal formulations were tested using either HSPC (Figure 1A-C) or DSPC (Figure 1D-F) as the base lipid. Both these lipids have high transition temperatures (T_m ~55°C) and are commonly used in liposomal products. Both were combined with cholesterol and PEGylated DSPE-lipid (3:1:1 w/w ratio) at an initial total lipid concentration of 16 mg/mL in ethanol. Both liposome formulations encapsulated $[NH_4]_2SO_4$ 250 mM (pH 5.5) and the microfluidic production parameters FRR and TFR were assessed. The ratio between the aqueous and the organic phase was shown to play a key role in the vesicle size of the produced liposomes (Figure 1A, D). With the HSPC formulation, varying the FRR from 1:1 to 5:1 allows for control of the particle size, decreasing from approximately 120 nm to 45 nm (Figure 1A). Furthermore, controlling the ratio between 1:1 (50% ethanol) and 2:1 (33%) allowed the vesicle size to be fine-tuned between 120 and 70 nm, with particle sizes reducing by approximately 10 nm as the ethanol content in the flow rate mix decreased by approximately 3% (Figure 1A). The ability to control vesicle size through controlling FRR, was also demonstrated with DSPC-based liposomes. Again by controlling the FRR between 1:1 to 5:1, particle sizes between 120 nm to 45 nm could be produced (Figure 1D). In all cases, the PDI was below 0.2. From these results, we selected the FRR 1.5:1 for further testing, as the produced particle size and polydispersity correspond to the CQA of the originator product Doxil®/Caelyx® (particle sizes between 80 - 100 nm and PDI <0.2).

To investigate the impact of production speed, the role of TFR on vesicle attributes was assessed and HSPC- and DSPC-based liposomes were manufactured at flow rates between 10 and 20 mL/min (Figure 1B and E respectively). As the production flow rate is increased, no significant ($p > 0.05$) impact on vesicle size for both formulations tested was observed. This confirmed the production speed can be increased without modifying the physicochemical characteristics of the product.

In order to identify the initial total lipid concentration operating range, a range of concentrations from 2.5 to 40 mg/mL (FRR 1.5:1; TFR 12 mL/min) were selected. At the lowest concentration, liposomal formulations exhibited significantly larger particle size compared ($p < 0.05$) to any of the other concentrations evaluated (110 nm and 95 nm for HSPC- and DSPC-based liposomes respectively) (Figure 1C,F). From 5 to 40 mg/mL, HPSC liposome sizes were all in the specified range of 80 to 100 nm, PD <0.2 (Figure 1C,F). Preparation of the formulation at 40 mg/mL at a FRR of 1.5:1, corresponds to a final concentration of 16 mg/mL (which is the concentration found in the marketed product). Initial lipid concentration is often dictated by the maximum solubility of the lipid(s) in the given solvent; however, during buffer exchange liposome suspension concentrations can be controlled and concentration-steps can be incorporated within the purification process.

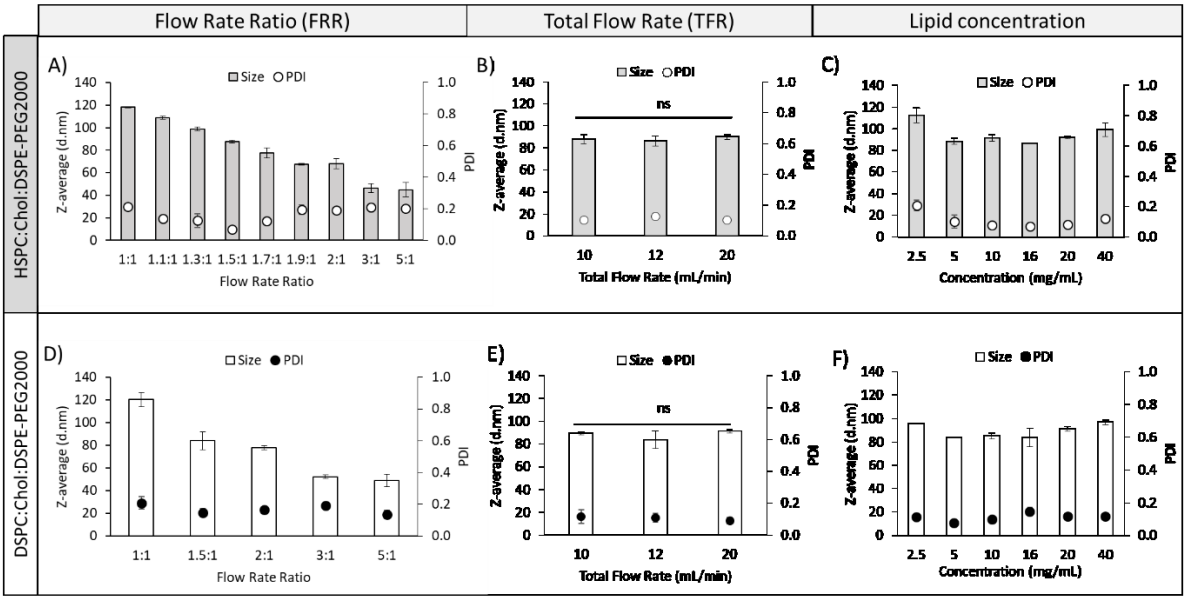


Figure 1. *Effect of the microfluidic process parameters on the physicochemical characteristics of HSPC:Chol:DSPE-PEG2000 and DSPC:Chol:DSPE-PEG2000 liposome formulations prepared in ethanol at 3:1:1 w/w. (A,D) The effect of flow rate ratio (FRR), (B,E) total flow rate (TFR), and (C,F) initial lipid concentration for HSPC:Chol:DSPE-PEG2000 (A,B,C) and DSPC:Chol:DSPE-PEG2000 (D,E,F) was measured. Unless varied, a FRR 1.5:1 and TFR 12 mL/min was used. Results represent the mean of at least 3 independent batches and the standard deviation is plotted as error bars*.

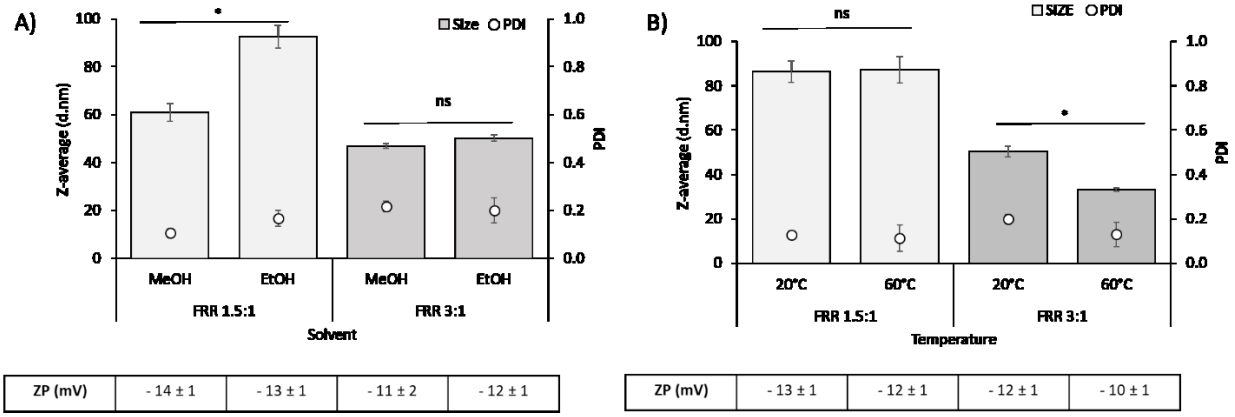
The effect of the FRR on the size of other nanoparticles has been previously reported by our group and others (Cheung and Al-Jamal, 2019; Forbes et al., 2019; Jahn et al., 2010; Kastner et al., 2014; Roces et al., 2020; Roces et al., 2019; Zizzari et al., 2017). For example, Forbes et al. demonstrated that non-PEGylated DSPC:Chol liposomes produced at 1:1 FRR resulted in the largest particle size compared to 3:1 and 5:1 FRR. Cheung and Al-Jamal (2019) recently produced PEGylated DOPC/DSPC:Chol:DSPE-PEG2000 and showed that the main parameter dictating the physicochemical characteristics of the liposomal product was the FRR. This control is due to the solvent mixing ratio controlling the rate of nanoprecipitation and formation of vesicle bilayers, with higher solvent ratios reducing the rate of precipitation and hence promoting larger vesicle formation. Low initial lipid concentrations may also impact on the rate of formation of vesicle bilayers, with slower formation and larger vesicles forming (Forbes et al., 2019). Similar to previous studies (Cheung and Al-Jamal, 2019; Forbes et al., 2019), we also show that the production speed did not impact the particle size, nor the polydispersity of the final product demonstrating that mixing and rate of nanoprecipitation remains uniform across the production speeds. Particle size and particle size distribution are CQA that must be evaluated for the production of nanosimilars or follow-on products. These characteristics influence the pharmacokinetics (including adsorption, biodistribution, degradability and cellular uptake) of the product. Although nanosimilars may have the same average particle size, they can still differ in the polydispersity (Astier et al., 2017), yet, the FDA's guidance for liposomal products recognises both these factors as CQA, although it does not indicate a satisfactory polydispersity value (FDA, 2018). However, generally a PDI of <0.2 is used as a benchmark.

3.1.2 The choice of flow rate ratio also controls the impact that solvent selection and operating temperature has on liposome size.

We further evaluated the effect of the manufacturing parameters on the HSPC-based liposome formulations by considering the choice of organic solvent used for lipid dissolution and the temperature of production (Figure 2). In terms of solvent selection, methanol and ethanol were used as both organic solvents are water miscible and therefore, can be used for the microfluidic production of liposomes; however, for large-scale manufacture ethanol would be the preferred option due to their relative safety profiles. When PEGylated liposomes were produced at 1.5:1 FRR, the particle size was dependent on the solvent used; liposome size significantly ($p < 0.05$) increased from 60 nm (methanol; MeOH) to 90 nm (ethanol; EtOH) (Figure 2A). Irrespective of the solvent used, the particle size distribution (PDI) and the zeta potential values were similar with the liposome suspensions being highly homogeneous (PDI ≤0.2), and slightly negative (˜-12 mV) (Figure 2A). In contrast, when liposomes were produced at 3:1 FRR, the physicochemical characteristics of the PEGylated liposomes

remained unaffected regardless of the use of methanol or ethanol as solvent (size range of 47 – 50 nm, PDI <0.2 and zeta potential of -10 to -12 mV; Figure 2A). Previous studies on microfluidic production of liposomes by Webb et al. demonstrated that the choice of organic solvent is an important consideration as it can impact on the size of the produced liposomes (Webb et al., 2019). Furthermore, Webb et al. showed that the addition of >14 w% PEG into DSPC:Chol formulation, negates the solvent effect and therefore, the particle sizes remained the same for both solvents (2-propanol and EtOH) tested. However, below 14 w% PEG, the choice of solvent affected the characteristics of the vesicles produced (Webb et al., 2019). Here, we demonstrated that, in agreement with Webb et al., when 3:1 FRR is applied (equivalent to 25% volume solvent), the effect of the solvent can be negated due to the existence of PEGylated lipid in our formulation (20 w%). However, when higher solvent concentrations are present (e.g. 1.5:1 FRR, equivalent to 40% solvent in the mixture) PEGylated formulations are sensitive to solvent selection. Therefore, the effect of solvent is both formulation and FRR (solvent concentration) dependent.

Previously, in our laboratory setting we have demonstrated that liposomes containing high transition temperature lipids (e.g. DSPC) can be manufactured at ambient temperature using microfluidics without impacting on vesicle size (Forbes et al., 2019). To confirm this applied to HSPC-based liposome formulations, these liposomes were manufactured at 18 - 20°C (ambient temperature) and 60°C (slightly above HSPC transition temperature; T_m 55°C) at both 1.5:1 and 3:1 FRR. As shown in Figure 2B, when prepared using 1.5:1 FRR, vesicle size was not dependent on temperature. However, formulations prepared at 3:1 FRR did show a significant difference ($p < 0.05$) in terms of vesicle size (from 50 to 33 nm for 20°C and 60°C respectively). Previously, Forbes et al. (Forbes et al., 2019) evaluated the effect of the temperature (from 20°C to 60°C) on liposomes prepared using high transition lipids (DSPC) containing different amounts of cholesterol within the formulation. Their studies showed that DSPC:Chol liposomes could be produced at room temperature and that the main factor controlling the vesicle size of the DSPC:Chol liposomes was the cholesterol content, whereas the temperature was shown to not affect the vesicle size (Forbes et al., 2019). We hypothesise that the addition of DSPE-PEG2000 into our formulation contributed to the reduction in the particle size when liposomes were produced at 3:1 FRR. For the 1.5:1 FRR, the higher solvent volume in the formulation, might negate the effect of the temperature during the manufacture of HSPC:Chol:DSPE-PEG2000 liposomes.



ZP (mV)	- 14 ± 1	- 13 ± 1	- 11 ± 2	- 12 ± 1

ZP (mV)	- 13 ± 1	- 12 ± 1	- 12 ± 1	- 10 ± 1

Figure 2*. Impact of the (A) solvent selection and (B) processing temperature on the physicochemical characteristics (particle size – bars; polydispersity – open circles; zeta potential - values) of HSPC:Chol:DSPE-PEG2000 (final concentration 4 mg/mL) prepared at FRR 1.5 and 3:1 and TFR 12 mL/min. Results represent the mean of at least 3 independent batches and the standard deviation is plotted as error bars. Significant differences are shown as *p <0.05.*

3.2 Doxorubicin loading is affected by extrinsic and intrinsic manufacturing properties

After evaluation of the process and formulation parameters, the next step was the active loading of doxorubicin (Figure 3). However, prior to doxorubicin encapsulation into the PEGylated liposomes, the tangential flow filtration washing cycle for the removal of non-encapsulated doxorubicin was optimised to allow drug loading to be quantified (Figure 3A). Concentrations of 0.25, 0.5 and 2 mg/mL free doxorubicin were used for tangential flow filtration cycle optimisation. A minimum volume of 2 mL was used in order to be above the tangential flow filtration system void volume (in our case 1.5 mL). By measuring the absorbance in the collected filtrate aliquots, we determined that for low doxorubicin concentrations (0.25 and 0.5 mg/mL) 6 diafiltration cycles (12 mL) were enough to remove the non-encapsulated drug. For higher doxorubicin concentrations (2 mg/mL), which correspond to the original drug concentration in the originator product, 13 diafiltration cycles (26 mL) ensured removal of free drug (Figure 3A).

3.2.1 Extrinsic manufacturing properties: doxorubicin loading time/temperature, external buffer choice and particle size impact on the doxorubicin encapsulation efficiency.

Given our ability to remove non-entrapped doxorubicin, the next steps were to determine the time and temperature required for the encapsulation of doxorubicin into the liposomes by active loading. Due to the transmembrane pH gradient between the internal aqueous core (acidic) and the external buffer environment (pH 6.5 to 7.5) of the liposomal formulation, amphipathic weak bases cross the

lipid bilayer and crystallise in the interior of the liposome mainly due to ionisation of the molecule (doxorubicin is protonised at acidic pH). By this means, the drug is trapped within the liposomes. Therefore, liposomes are prepared encapsulating $[NH_4]_2SO_4$ 250 mM (pH 5.5) and after replacing the external $[NH_4]_2SO_4$ or adjusting to physiological pH, doxorubicin is added into the extraliposomal media. A pH gradient across the liposome membrane is established due to free ammonia created from the dissociation of $[NH_4]_2SO_4$ into 2 ammonium cations and 1 sulfate anion. Thus, doxorubicin, which is uncharged at physiological pH, is able to cross the liposomal membrane and crystallises in the aqueous interior of the liposome due to self-association and association with the remaining sulfate anion (Barenholz, 2001). By this means, doxorubicin is efficiently encapsulated into the liposomes, with more than 90% of the drug encapsulated as doxorubicin-sulfate crystals, which grants higher stability of the drug within the liposome core due to the lack of osmotic effect of the crystal. Thus, in Figure 3B and C we evaluated the extrinsic factors (loading time and temperature, choice of external buffer and the production parameter flow rate ratio) influencing doxorubicin loading into HSPC:Chol:DSPE-PEG2000. Figure 3B shows the time needed to achieve above 90% EE (drug-to-lipid ratio of 1:8 w/w) at three loading temperatures: ambient temperature (25°C), below the bilayer transition temperature (40°C) and above the transition temperature (60°C). When prepared with PBS as the external buffer, less than 20% EE was achieved at room temperature after 180 min (Figure 3B). When the temperature was increased to 40°C, after 2 hours ≥90% drug loading was achieved and this was reduced to 10 min when the liposomes and drug were incubated at 60°C (Figure 3B). Using Histidine-Sucrose (His-Suc, pH 6.5) as the external buffer (which is the external buffer used in the marketed formulation), a longer time (30 min) was needed to achieve ≥90% EE at 60°C (Figure 3B). These differences in loading time between the two buffers may be related to the difference in pH, viscosity and/or osmotic pressure of the external medium which result in a different gradient magnitude and/or rate of diffusion. In general, the majority of studies use ≥60°C temperature for doxorubicin loading; Zucker et al. depicted a frequency bar chart demonstrating that more than 60 studies used 60°C for the remote loading of doxorubicin (Zucker et al., 2009). Irrespective of the temperature, time and external buffer used, the physicochemical characteristics of the doxorubicin loaded PEGylated liposomes remained unaffected and independent of these parameters (Figure 3C). However, as would be expected the zeta potential differs significantly ($p < 0.05$) between doxorubicin-loaded liposomes formulated using PBS and His-Suc (-12 mV compared to -35 mV respectively; Figure 3C).

Given that FRR was shown to impact on particle size (Figure 1A), the impact of this particle size control on doxorubicin loading was also tested. Figure 3D shows that liposome particles manufactured from 1:1 to 1.7:1 FRR with corresponding particle sizes of 110 nm down to 85 nm are able to achieve 90%

and above doxorubicin loading. However, when the FRR was increased to 2:1 or above, the smaller liposomes formed (approx. 65 nm) showed significantly ($p < 0.05$) lower encapsulation efficiencies, down to 40% (Figure 3D), presumably due to the low liposome internal volume, which can result in lower drug loading (Ponce et al., 2006).

Encapsulation of doxorubicin within liposomes can be carried out by passive or active loading methods. Generally, passive methods result in low encapsulation efficiencies (≤40%) whereas active loading results in a very efficient method for high doxorubicin encapsulation, able to achieve near 100% and for this reason it is commonly adopted (e.g. (Barenholz, 2001, 2012; Haran et al., 1993; Silverman and Barenholz, 2015; Zucker et al., 2009)). EE% and drug retention are both CQA since liposomes should be able to carry enough doxorubicin concentration to the tumour cells and also, reduce the toxicity of the free doxorubicin (Ponce et al., 2006).

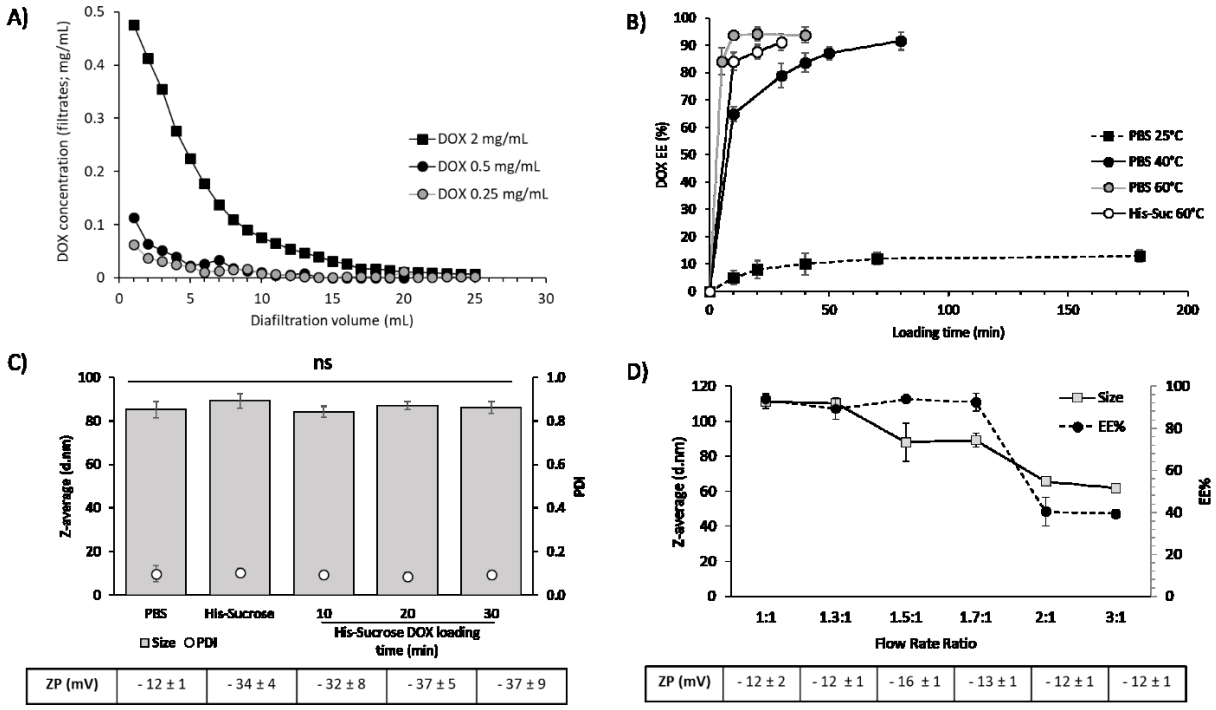


Figure 3. *Remote loading of doxorubicin (DOX) into liposomes. (A) Optimisation of the tangential flow filtration cycle for doxorubicin removal using free doxorubicin at 0.25, 0.5 and 2 mg/mL. (B) Effect of loading time and temperature using HSPC:Chol:DSPE-PEG2000 (10 mg/mL initial lipid concentration in ethanol, FRR 1.5:1 and TFR 12 mL/min) in ethanol with PBS (pH 7.4, 10 mM) or histidine-sucrose buffer (10 mM, 10%, pH 6.5) as external buffer. (C) Particle size (bars), polydispersity (open circles; PDI) and zeta potential (table) of HSPC:Chol:DSPE-PEG2000 liposomes (10 mg/mL initial lipid concentration in ethanol) at FRR 1.5:1 and TFR 12 mL/min using PBS or His-Suc as external buffer, before and after doxorubicin loading (from 10 to 30 min at 60°C). (D) Particle size and encapsulation efficiency (EE%) of the HSPC:Chol:DSPE-PEG2000 liposomes formulated with ethanol and $[NH_4]_2SO_4$ 250 mM at 1:1 to 3:1 FRR, final lipid concentration of 4 mg/mL, PBS external buffer and loaded during 10 min at 60°C. Results represent the mean of at least 3 independent batches and the standard deviation is plotted as error bars. Significant differences are shown as *p <0.05.*

3.2.2 Intrinsic manufacturing properties: Initial solvent choice impacts on drug loading.

Based on the previous results, we identified that particle size was a critical attribute in the doxorubicin loading due to the correlation to internal volume (Barenholz, 2012). However, given we had seen that FRR and solvent selection impact on vesicle size, we further explored potential critical formulation factors that may influence doxorubicin loading efficiency. Therefore, we assessed the doxorubicin loading efficiency of both HSPC:Chol:DSPE-PEG2000 and DSPC:Chol:DSPE-PEG2000 liposomes formulated using different solvents (Figure 4). With HSPC-based formulations, we screened liposomes prepared in methanol, ethanol or a combination (50:50 and 25:75 v/v, methanol:ethanol) (Figure 4A to C). Figure 4A shows a trend of increasing vesicle size as we move from methanol to ethanol, with vesicles formed using methanol being significantly ($p < 0.05$) smaller than those formed in ethanol (approximately 60 nm when formed using 100% methanol compared to appoximately 90 nm using 100% ethanol; Figure 4A). When these vesicles were loaded with doxorubin, there was no significant increase in size (Figure 4A). Across the solvent range tested there was no signficant difference in zeta potential (Figure 4B). When these vesicles were subjected to active loading with doxorubin, drug loading was notably different; Figure 4C shows that the encapsulation efficiency calculated for these particles was approximately 20, 30, 60 and 94% for the formulations prepared using methanol (100%), methanol:ethanol (50:50 and 25:75 v/v %) and ethanol (100%), respectively (Figure 4C). The effect of solvent selection on particle size and drug loading was also confirmed with DSPC-based liposomes (Figure 4D-F). DSPC:Chol:DSPE-PEG2000 liposomes produced using either methanol or ethanol displayed particle sizes of 60 and 80 nm respectively ($p < 0.05$), with EE% of 20% and near 80% EE ($p < 0.05$) (Figure 4D-F). Therefore, whilst particle size may be a contributing factor in drug loading, the large differences in drug loading noted for liposomes prepared in methanol vs ethanol (20% vs 90% respectively) do not map to the differences in particle size (60 vs 90 nm respectively), given that 60 nm vesicles formed with ethanol can achive 40% loading (Figure 2D). Therefore, we concluded that both, particle size and choice of solvent affect doxorubicin encapsulation efficiency and potentially low levels of residual solvent may be influencing the permeability of the bilayer and possibly reducing the pH gradient.

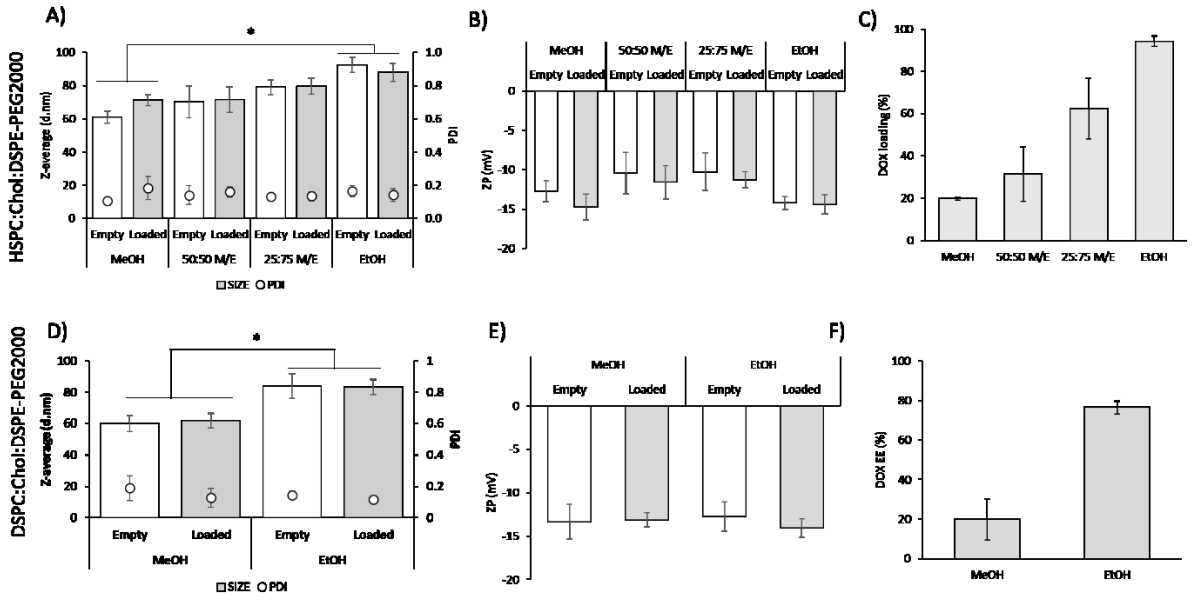


Figure 4. *Effect of the solvent adopted using liposomal manufacturing on doxorubin active loading. Empty and doxorubicin-loaded HSPC:Chol:DSPE-PEG2000 (A,B,C) and DSPC:Chol:DSPE-PEG2000 (D,E,F) liposomes manufactured using microfluidics at FRR 1.5:1 and TFR 12 mL/min at an initial lipid concentration of 10 mg/mL. (A,D) Particle size (bars) and polydispersity (PDI; open circles), (B,E) zeta potential values and (C,F) encapsulation efficiency (EE%). Results represent the mean of at least 3 independent batches and the standard deviation is plotted as error bars. Significant differences, where not obvious, are shown as *p <0.05.*

3.3 Down-streaming process for the preparation of liposomes: Pharmaceutical quality monitoring

Using the above optimised protocols, down-stream processing was incorporated into the manufacture of HSPC:Chol:DSPE-PEG2000 liposomes and the process assessed in terms of particle size, polydispersity, drug recovery and liposome yield (Figure 5A-C). Figure 5A shows the particle size and polydispersity of the HSPC:Chol:DSPE-PEG2000 formulation through the manufacturing process: manufacture in ethanol encapsulating $[NH_4]_2SO_4$ 250 mM using microfluidics, followed by solvent removal, external buffer exchange to PBS (purification) and concentration from 4 mg/mL up to 16 mg/mL. Then doxorubicin loading (10 min at 60°C) and finally sterile filtration (0.22 µm filter) (Figure 5A). After each stage, the vesicle size and polydispersity remained consistent (80 - 95 nm and 0.1 respectively). Figures 5B and C show the liposome encapsulation efficiency and liposome yield (in terms of lipid recovery and number of particles) before and after sterile filtration; results showed that HSPC:Chol:DSPE-PEG2000 can be sterilised at 16 mg/mL with near 100% drug and liposome recovery.

Given the production process was now standardised, we assessed the stability of the product. PEGylated liposomes encapsulating doxorubicin were stored at 4°C for 6 months and the vesicle characteristics measured at selected intervals (Figure 5D-G). The vesicles retained their particle size (80 - 95 nm) across the duration of the study (180 days) and the polydispersity remained below 0.2

(Figure 5D). Figure 5E shows the average intensity plots of each size measurement from day 0 to day 180. The zeta potential was also retained across the time-course of the study (-9 to -15 mV; Figure 5F). Doxorubicin loading was calculated at the end of the study, and results showed that 91.5 ± 3% doxorubicin remained entrapped within the liposomes after 6 months (Figure 5G).

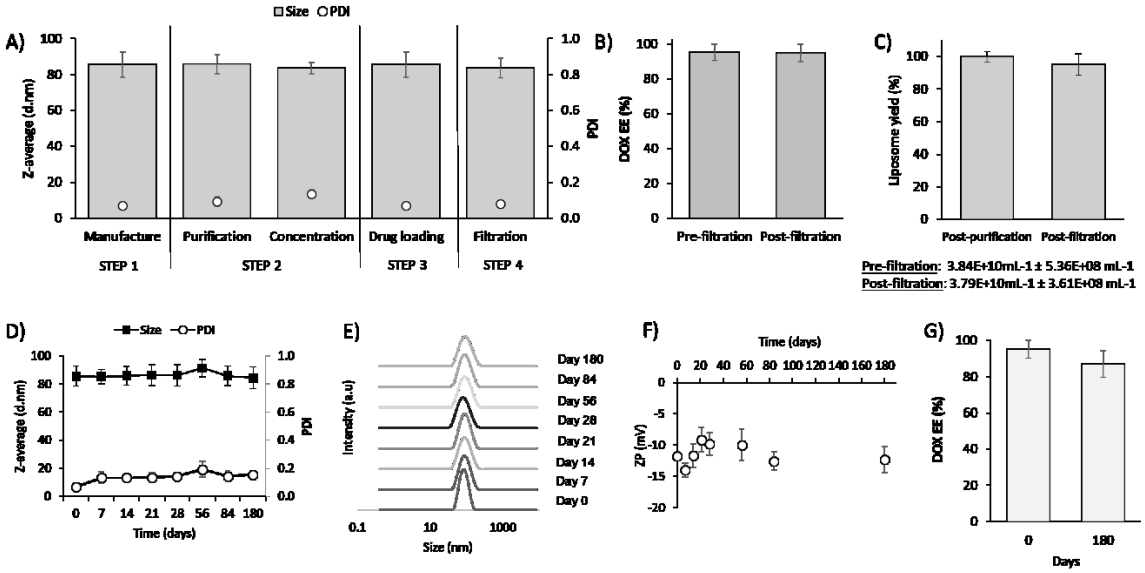
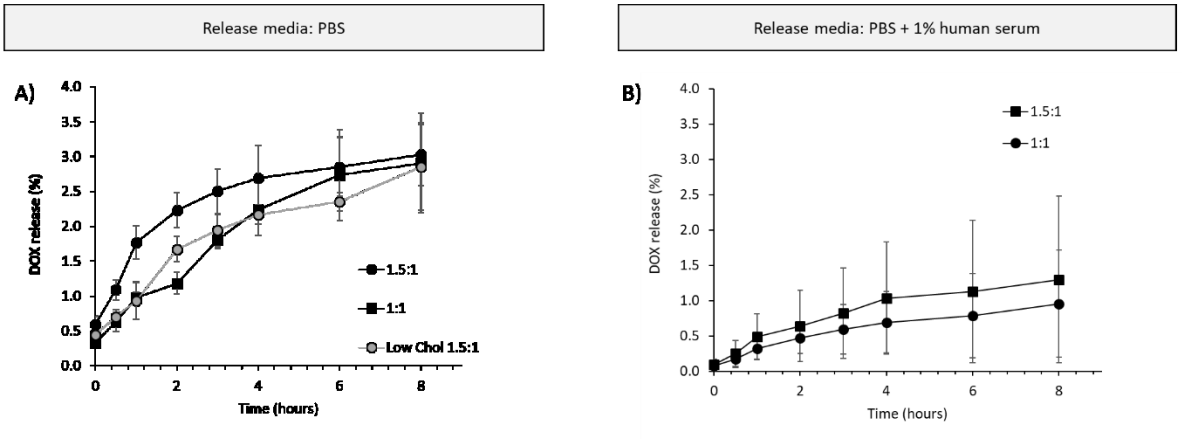


Figure 5*. Manufacture and down-stream process of HSPC:Chol:DSPE-PEG2000 liposomes active-loaded with doxorubicin. (A) Physicochemical characterisation of HSPC:Chol:DSPE-PEG2000 FRR 1.5:1 TFR 12 mL/min at different processing stages: manufacture, purification, concentration, drug loading and filtration. (B) Drug recovery and (C) liposome and particle recovery after syringe filtration (filter 0.22 µm) of the doxorubicin-loaded HSPC:Chol:DSPE-PEG2000 formulation TFR 12 mL/min and FRR 1.5:1 (16 mg/mL final lipid concentration). A stability test of the doxorubicin-loaded liposomes at 4°C was carried out for 6 months and the physicochemical characteristics of the particles measured at selected time points: (D) particle size and polydispersity, (E) intensity plots of the particles and (F) zeta potential. (G) Doxorubicin loading as percentage of the initial amount loaded on day 0 of the experiment and day 180. Results represent the mean of at least 3 independent batches and the standard deviation is plotted as error bars.*

Conservation of the physicochemical stability of the liposomal system over time is critical and establishing a product shelf-life is key. Indeed, studies on the effect of the liposomal particle size on tumour biodistribution studies showed similar tumour distribution on particles from 80 to 150 nm diameter, whereas particles higher that 240 nm diameter exhibited differences (Haran et al., 1993). According to the European review of Caelyx®, variation of the liposomal particle size between 100 to 150 nm does not have any effect on the antitumour efficacy of the product (Burgess et al., 2004). Within the Caelyx® liposome formulation, it has been reported that the ammonium sulfate gradient helps with the stabilisation of doxorubicin for extended storage periods (more than 6 months) due to the doxorubicin-sulfate crystals (Haran et al., 1993).

3.4 *In vitro* doxorubicin drug release assay

Considering that the pharmacokinetic and pharmacodynamic characteristics of the PEGylated doxorubicin formulation depend on several CQA including vesicle size, drug loading and drug release, an *in vitro* doxorubicin release study should be performed to assess the doxorubicin and lipid bilayer physical state and to confirm drug retention under physiological conditions. Indeed, Smith et al. reported lower doxorubicin tumour concentration (almost half) after administration of Lipodox® in human ovarian cancer mouse model compared to the originator product Doxil®/Caelyx® despite both having similar drug loading (Smith et al., 2016). This correlates to the reduced efficacy of Lipodox® compared to the originator product in the mouse model as well as in the treatment of ovarian cancer (Berger et al., 2014; Smith et al., 2016). Therefore, in addition to drug loading, drug retention/release is an important attribute to consider. However, there is no standard *in vitro* test to quantify doxorubicin loss from liposomes. We know that doxorubicin must remain within the liposomes and not leak out when circulating in plasma, so to probe this, we performed a release study using dialysis in PBS and PBS containing 1% human serum at 37°C (Figure 6A and B). To consider if this test was discriminatory, formulations were also prepared with low (25% weight less compared to marketed formulation) cholesterol concentrations, given that cholesterol is a key factor in drug release from liposomes (Gregoriadis and Davis, 1979; Kirby et al., 1980). Results showed that doxorubicin release is minimal under these conditions (up to 8 h), in line with previous studies (Niu et al., 2010). PEGylated liposomes released approximately 3% of the encapsulated doxorubicin after 8 h when incubated in PBS at 37°C, independent of the cholesterol content, particle size (between 80 to 110 nm) or microfluidic process parameter applied (Figure 6A). When doxorubicin-loaded liposomes were evaluated in PBS including human serum, these liposomes released significantly less ($p < 0.05$) than in PBS, releasing only 1.5% in 8 hours (Figure 6B). These low release rates are expected and in line with *in vivo* studies in animals and humans (Barenholz, 2012; Gabizon et al., 1994; Jiang et al., 2011). However, these studies also show that this protocol does not offer suitable discriminatory power given that the release from low cholesterol content liposomes was not significantly different to the other formulations tested.



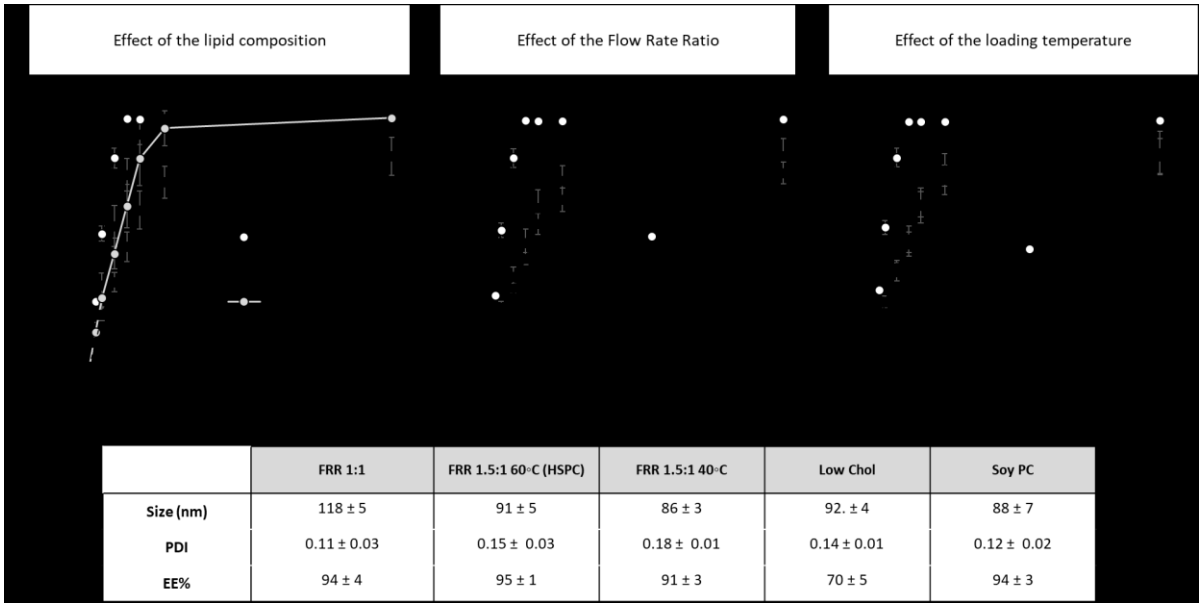
Before dialysis | After dialysis

C)

	FRR 1:1	FRR 1.5:1	Low Cholesterol	FRR 1:1	FRR 1.5:1	Low Cholesterol
Size (d.nm)	111. ± 4	87 ± 6	93 ± 3	108. ± 3	91 ± 14	93 ± 4
PDI	0.2 ± 0.04	0.15 ± 0.03	0.14 ± 0.01	0.28 ± 0.07	0.16 ± 0.07	0.14 ± 0.01
ZP (mV)	-13 ± 1	-14 ± 1	-14 ± 1	-13 ± 1	-14 ± 1	-13 ± 1

Figure 6*. Release profiles of doxorubicin-loaded liposomes. Different liposome formulations were incubated in (A) PBS media and (B) PBS + 1% human serum at 37°C. (C) Physicochemical characteristics of the liposomal formulations tested before and after the release study. Results represent the mean of at least 3 independent batches and the standard deviation is plotted as error bars.*

A recent paper from Yuan et al. (Yuan et al., 2017) developed a USP-4 apparatus method to distinguish between manufacturing techniques (homogenisation and extrusion) and between formulation parameters (size and lipid choice). Here, we followed this method and initially evaluated the effect of the lipid composition (SoyPC, HSPC and HSPC with 25% weight less cholesterol) to confirm if the model was discriminatory. Then, we applied the protocol to consider the effect of the FRR (1:1 vs 1.5:1) and the effect of the doxorubicin incubation temperature (60°C vs 40°C) on the doxorubicin release profiles (Figure 7). Results showed that this method can indeed differentiate between different liposomal compositions (f_2 <50), with liposomes containing either SoyPC or low cholesterol content releasing faster (approx. 100% in 6 h) than HSPC:Chol:DSPE-PEG2000 liposomes (Figure 7A). Therefore, this protocol was adopted to test the effect of the liposomal size/FRR by comparing liposomes prepared at different FRRs (Figure 7B). The results showed no significant difference (f_2 >50) in release rates between liposomes produced at 1:1 FRR (120 nm) and those produced at 1.5:1 FRR (90 nm). Similarly, there was no significant difference (f_2 >50) in release profiles from liposomes loaded with drug using an incubation temperature of 40°C or 60°C (80% drug release in 24h) (Figure 7C). The characteristics of each of the formulations tested is summarised in Figure 7D.



	FRR 1:1	FRR 1.5:1 60°C (HSPC)	FRR 1.5:1 40°C	Low Chol	Soy PC
Size (nm)	118 ± 5	91 ± 5	86 ± 3	92. ± 4	88 ± 7
PDI	0.11 ± 0.03	0.15 ± 0.03	0.18 ± 0.01	0.14 ± 0.01	0.12 ± 0.02
EE%	94 ± 4	95 ± 1	91 ± 3	70 ± 5	94 ± 3

Figure 7*. Release studies using the USP-4 apparatus test assessing: (A) the lipid composition (HSPC:Chol:DSPE-PEG2000 vs SoyPC:Chol:DSPE-PEG2000 vs HSPC:LowChol:DSPE-PEG2000) (B) the influence of the FRR (1:1 vs 1.5:1) (C) and the incubation temperature (40°C vs 60°C) on the production of DOX-loaded PEGylated liposomes. (D) Physicochemical characteristics of the liposomal formulations tested before the release study. Results represent the mean of at least 3 independent batches and the standard deviation is plotted as error bars.*

To control the pharmaceutical quality of a product, *in vitro* release testing should be cost-effective and able to distinguish between important product quality changes, to better understand the drug release mechanism (Marques et al., 2019). The drug release assay previously developed Yuan et al. (Yuan et al., 2017) was shown to be discriminatory for different liposome formulations produced by microfluidics. Other factors such as the presence of lipid impurities, the state of the encapsulated drug (liquid vs crystal) or lower $[NH_4]_2SO_4$ concentration in the liposome interior would also contribute to a higher release rate (Ponce et al., 2006). However, no significant difference in release rate for liposomes produced at 1:1 vs 1.5:1 FRR or different loading temperatures was noted.

3.5 High-throughput is achieved using the scale-up system

After the optimisation of the microfluidic production parameters using a bench scale micromixer (up to 20 mL/min), the speed at which PEGylated liposomes were manufactured was evaluated using the scale-up microfluidic platform (at 12, 60 and 90 mL/min) (Figure 8). Liposome preparations were characterised for their particle size, size distribution, zeta potential, EE% and morphology (CryoTEM). Liposomal suspensions produced at 12 mL/min (bench-scale and scale-up), 60 mL/min and 90 mL/min (scale-up) all showed comparable physicochemical characteristics (85 - 100 nm) (Figure 8A-B), similar

drug loading (≥90%) (Figure 8C) and comparable morphology (Figure 8D). All formulations showed small particle size (80 – 100 nm), narrow size distribution (<0.2) and high reproducibility. Morphological characterisation showed small spherical unilamellar and very homogeneous liposomal populations for the empty formulations independent of the production system selected (Figure 8D).

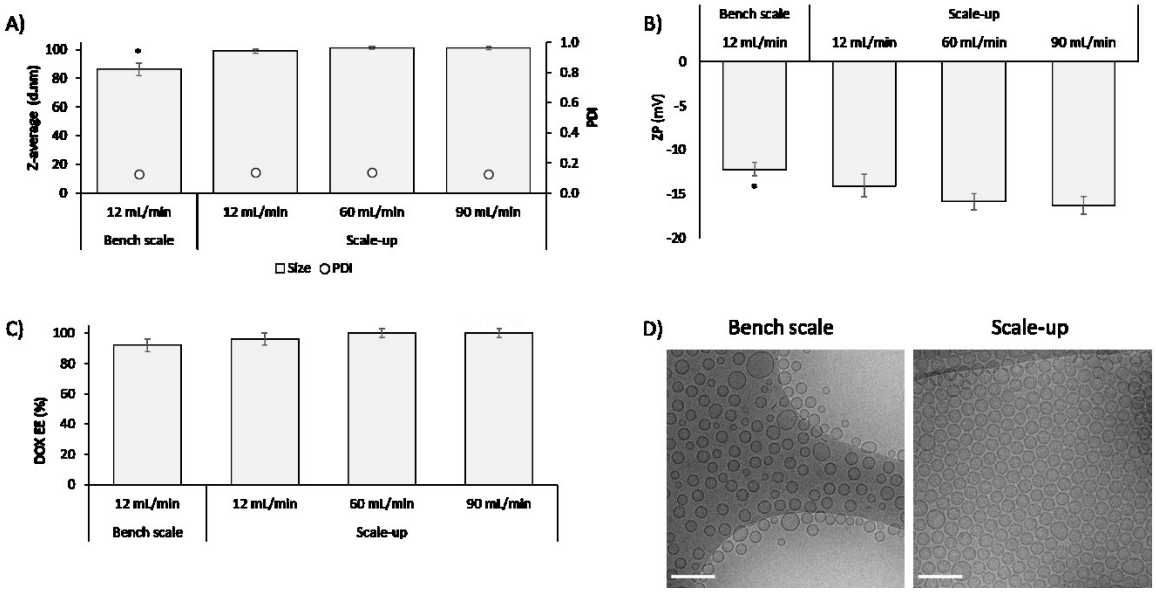


Figure 8*. Physicochemical characteristics of the HSPC:Chol:DSPE-PEG2000 (3:1:1 w/w) formulation manufactured using Precision NanoSystems microfluidic platform (Bench scale and pre-clinical scale: 12, 60 and 90 mL/min) at FRR 1.5:1. (A) Particle size (bars) and polydispersity (open circles), (B) zeta potential (ZP), (C) encapsulation efficiency (EE%) and (D) CryoTEM images of the empty formulation prepared using the bench scale and scale-up production technology (scale bar 200 nm). Significant differences are shown as *p <0.05.*

3.6 Liposomal formulations manufactured using the microfluidic platforms map to originator product.

The doxorubicin release profile from the HSPC:Chol:DSPE-PEG2000 formulations prepared using the bench scale and scale-up system (at 12, 60 and 90 mL/min) were compared to the originator product (Doxil®/Caelyx®). All formulations showed particle sizes between 80 to 100 nm. Due to supply issues with the originator product, we developed a 'reverse gradient method' release protocol, which allows us to discriminate between formulations using very low sample volume and concentration (Figure S2). We based this test on results from Silverman et al., where it is hypothesised that the PEGylated liposomes release doxorubicin within the tumour site due to the ammonia produced there by glutaminolysis (Silverman and Barenholz, 2015). The reported *in vitro* release study shows higher doxorubicin release using 50 mM $[NH_4]_2SO_4$ as release inducer in a normal saline medium buffered with 20 mM histidine (pH 7.3) at 37°C (Silverman and Barenholz, 2015). All products released 80 to 85% of the encapsulated drug released after 48 hours (Figure 9) and drug release profiles were not

significantly different (f_2 >50) across the production scales tested and compared to the originator product, demonstrating that the scale-up manufacturing process can be applied to produce a product mapped to the originator product. These results demonstrate the ability of producing from bench to clinic scale doxorubicin-loaded liposomes offering high loading and particle sizes using the same manufacturing process parameters.

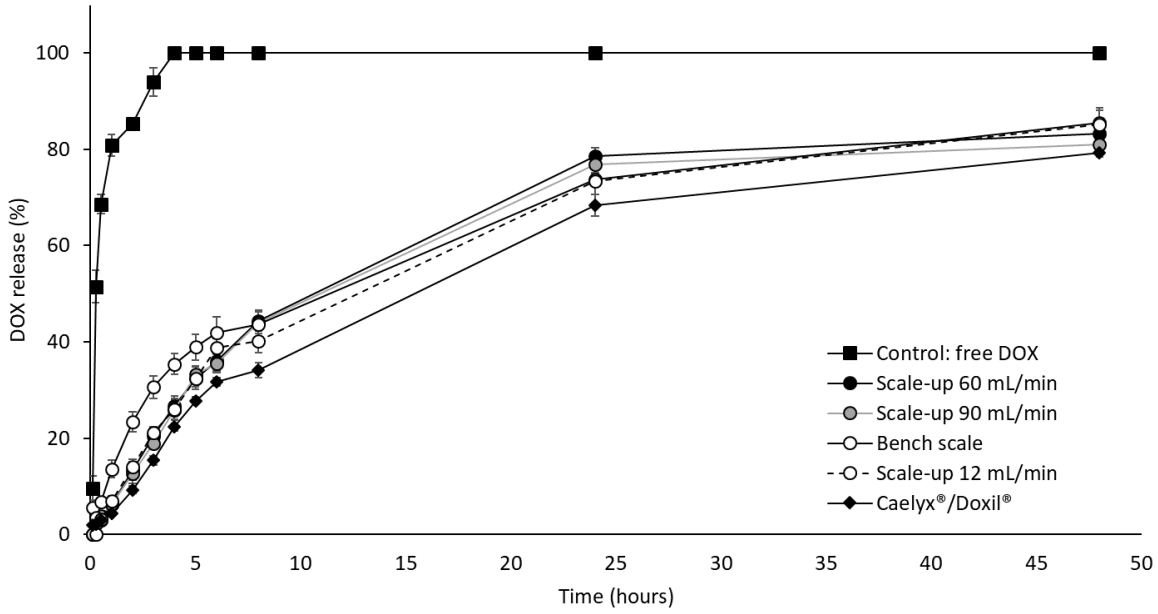
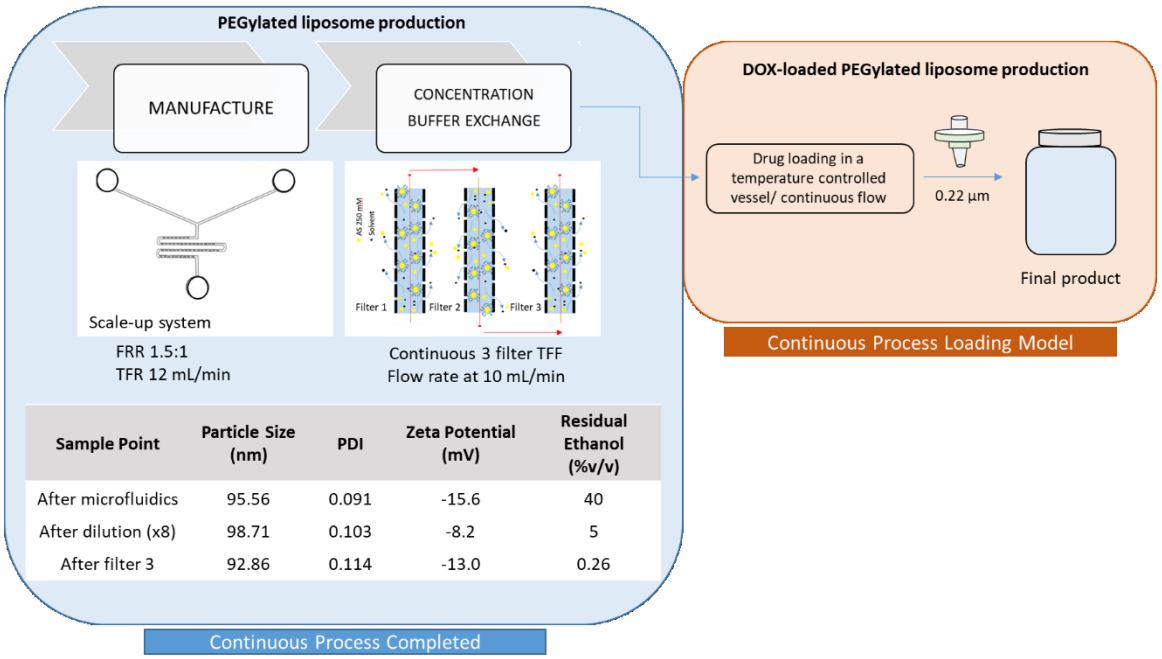


Figure 9*. Drug release profiles of doxorubicin-loaded HSPC:Chol:DSPE-PEG2000 (1.5:1 FRR) using the bench scale (12 mL/min) and scale-up platform (12, 60 and 90 mL/min). Drug release was assessed using dialysis and ammonium sulfate pH 6.6 as release inducer at 45°C. Results represent the mean of 3 batches and the standard deviation is plotted as error bars.*

We further developed a continuous manufacturing process for the production of PEGylated liposomes (HSPC:Chol:DSPE-PEG2000) encapsulating $[NH_4]_2SO_4$ 250 mM. By this means, a three-filter tangential flow filtration system was used after production of PEGylated liposomes at the optimised parameters: 1.5:1 FRR and 12 mL/min TFR using the scale-up microfluidic system (Figure 10). The physicochemical characteristics of the formulation (vesicle size, size distribution and zeta potential) were comparable after microfluidic production, dilution and buffer exchange. At the end of the process, PEGylated liposomes were approximately 95 nm, 0.1 PDI and -13 mV zeta potential. Quantification of the ethanol content within the formulation shown values below the ICH guidelines for residual solvent (<0.5% v/v) (Figure 10) and the product was stable for up to 2 months (data not shown). To move to a fully continuous process, the development of an additional temperature controlled vessel/feedline for the active-loading of drugs could be added along with online characterisation tools. However, currently there is no process analytical methods available for microbial contamination, so sterility assurance would need to be assured through the design and validation of the system.



Sample Point	Particle Size (nm)	PDI	Zeta Potential (mV)	Residual Ethanol (%v/v)
After microfluidics	95.56	0.091	-15.6	40
After dilution (x8)	98.71	0.103	-8.2	5
After filter 3	92.86	0.114	-13.0	0.26

***Figure 10**. Schematic representation of the continuous manufacturing pilot plan. Empty PEGylated liposomes with a pH gradient were successfully manufactured in a continuous manner using the scale-up microfluidic system attached to a three-filter tangential flow filtration system. Table shows the vesicle size, polydispersity, zeta potential and residual solvent content at each stage of the process. Further studies will focus on developing a continuous process for the active-loading of drugs.*

3.7 On-demand and just-in-time drug loading of liposomes

In addition to offering scale-independent and continuous manufacturing, microfluidic production also offers the ability for on-demand or just-in-time production. Given that a range of drugs can be loaded into liposomes using a pH gradient, our microfluidic production method can be used for just-in-time drug loading which could underpin personalised manufacturing. Just-in-time production reduces time within the production system, and potentially cuts costs and waste through a supply on demand strategy. To demonstrate the feasibility with our production method, we selected three amphipathic weak bases: vincristine (VIN), doxorubicin (DOX) and acridine orange (AO). HSPC:Chol:DSPE-PEG2000 liposomes encapsulating $[NH_4]_2SO_4$ 250 mM were produced using our optimised production process (1.5:1 FRR, 10 mg/mL initial total lipid in ethanol). For drug loading, the drug-to-lipid ratio, loading time and temperature were selected based on the results from Zucker et al (Zucker et al., 2009). Figure 11 shows the production of 50 mL batch of HSPC:Chol:DSPE-PEG2000 liposomes using the scale-up microfluidic platform; the particle size of the liposomes is approximately 100 nm, with a narrow size distribution. Aliquots of liposomes from this batch were loaded (>90%) with doxorubicin, vincristine or acridine orange and the physicochemical characteristics of the formulations remained unaffected.

These results suggest the potential for microfluidics to be used within a just-in-time manufacturing process.

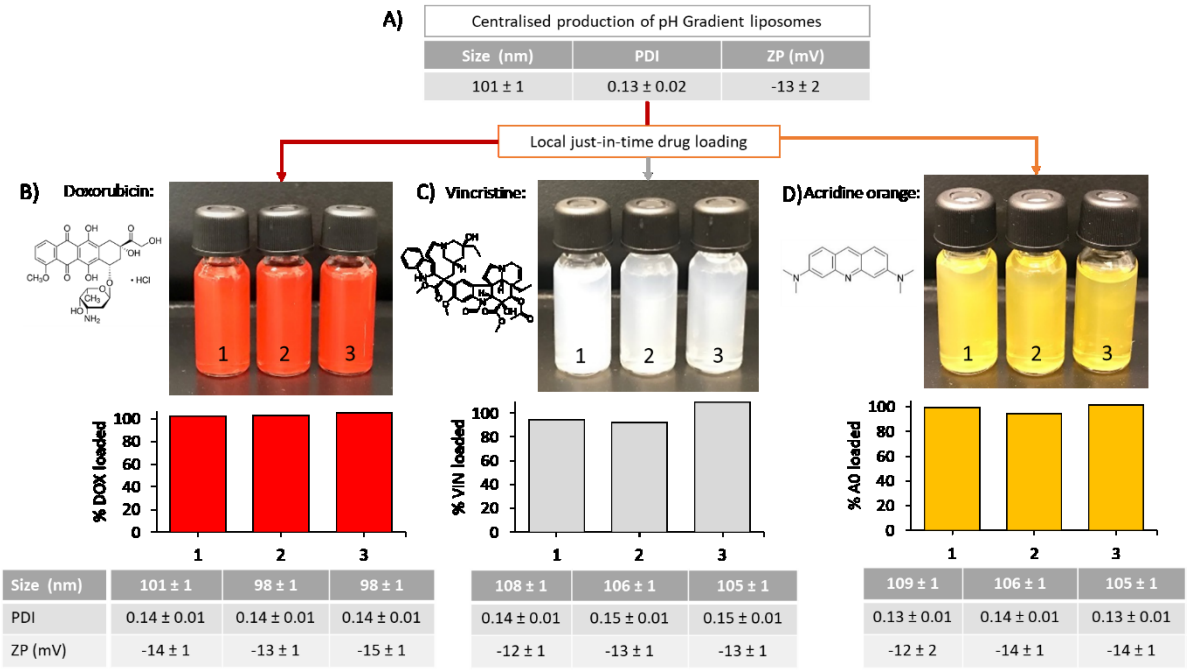


***Figure 11**. Just-in-time personalised medicine. (A) Particle size, polydispersity and zeta potential of 50 mL batch of HSPC:Chol:DSPE-PEG2000 liposomes using the scale-up microfluidic system (1.5:1 FRR, 12 mL/min).. Particle size, polydispersity, zeta potential (tables) and EE% (graphs) of the aliquots loaded with doxorubicin (DOX), vincristine (VIN) and acridine orange (AO) respectively.*

4. Conclusions

For nanomedicines to be more commonly exploited in global healthcare, more rapid and cost-effective manufacturing methods are needed. The rise of manufacturing techniques based on fluid control can directly address this. We have successfully demonstrated the ability of microfluidics to produce small unilamellar PEGylated liposomes with narrow particle size distribution to support tumour targeting. High drug encapsulation efficiencies (EE% ≥90) were achieved for all three drugs tested (doxorubicin, vincristine and acridine orange). In the case of doxorubicin, our results showed comparable physicochemical attributes and pharmaceutical quality criteria equivalence of the microfluidic PEGylated liposomes with the originator product Doxil®/Caelyx®. These criteria involved the measurement of liposomal size and size distribution, zeta potential, and the quantification of the unentrapped drug, particle stability, product sterilisation and drug release tests. Our outlined pilot model involved the manufacturing of PEGylated liposomes encapsulating $[NH_4]_2SO_4$ using microfluidics, concentration and establishment of the transmembrane gradient using tangential flow filtration, loading of the drug product via active loading and sterilisation of the end-product via

filtration. Furthermore, the degree of size-tuning offered by the microfluidics process provides the ability to create liposomal formulations for active loading of drugs in a scale-independent manner or in a just-in-time manufacturing setting.

CRediT authorship contribution statement

Carla B. Roces: data curation, methodology, formal analysis, writing, review, editing and visualisation; **Emily C Port:** data curation, methodology, writing, review, editing and visualisation; **Nikolaos N Daskalakis:** data curation, methodology, writing, review, editing and visualisation; **Julie A Watts:** data curation, methodology, writing, review, editing and visualisation; Jonathan Aylott: writing, review, editing and visualisation; **Gavin Halbert:** methodology, formal analysis, writing, review, editing, visualisation and supervision; **Yvonne Perrie:** Project administration, funding acquisition, conceptualization, methodology, formal analysis, writing, review, editing, visualisation and supervision.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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