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Research Paper

Scalable solvent-free production of liposomes

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Abstract

Objectives A major challenge faced with the manufacture of liposomes is the high volumes of organic solvents used during manufacturing. Therefore, we have implemented an organic solvent-free production method for drug-loaded liposomes and demonstrated its applicability with both aqueous core-loaded and bilayer-loaded drugs.

Methods Liposomes were produced by high shear mixing dry powder lipids with an aqueous buffer, followed by down-sizing using a Microfluidizer processor. Liposomes were purified via tangential flow filtration and characterised in terms of size, polydispersity index, zeta potential and drug loading.

Key findings Doxorubicin-loaded PEGylated liposomes can be manufactured using this solvent-free method with particle sizes of 100–110 nm, low polydispersity index (PDI) (<0.2) and high drug loading (97–98%). If required, liposomes can be further down-sized via microfluidic processing without impacting drug loading. Similar results were achieved with non-PEGylated liposomes. With bilayer-loaded amphotericin B liposomes, again liposomes can be prepared within a clinically appropriate size range (100–110 nm in size, low PDI) with high drug loading (98–100%).

Conclusions We apply a simple and scalable solvent-free method for the production of both aqueous core or bilayer drug-loaded liposomes.

Introduction

Liposomes have been extensively investigated for the delivery of both hydrophobic and hydrophilic drugs due to their ability to improve drug efficacy through targeting.[1] The number of liposomal approvals (Table 1) continues to grow in the United States (US)[2] and European Union (EU).[3] Furthermore, with many of the patents related to liposomal products expiring, there has been a rapid increase in the number of generic liposome products approved.[4] Table 2 summarises the rise in generic versions of the most successful and well-established products such as Caelyx/Doxil (PEGylated liposomal doxorubicin) and AmBisome (liposomal amphotericin B).

In the production of liposomes, both at the bench-scale and within large-scale production, organic solvents are commonly used, with examples outlined in Table 3. At the bench-scale, the thin film hydration method remains the most widely adopted method to manufacture liposomes and it is based on the dissolution of the lipid components with or without a drug in an organic solvent. The solvent is subsequently evaporated by rotary evaporation, followed by hydration of the film using an aqueous buffer. Other methods to produce liposomes include reverse-phase evaporation and ethanol injection.[5] When considering the choice of solvent, safe handling, removal and disposal is a key consideration as organic solvents can be associated with chronic health effects, especially halogenated solvents[6] and maximum allowable concentration limits for solvents within formulations are defined by the International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use (ICH) guidelines.[7] Solvents are grouped into four classes as per the ICH Q3C guidelines. Class 1 solvents are known as human carcinogens and suspected to be hazardous to the environment. Class 2 solvents (chloroform and methanol) should be limited as they are possible causative agents of irreversible toxicity. The acceptable concentration limit for chloroform and methanol is 60 and 3000 ppm, and the exposure limit is 0.6 and 30 mg/day, respectively. Ethanol is class 3 with a

Table 1 Marketed liposomal products, first approval and expected patent expiry dates[30,31]

Brand name	Drug	Indication	First approval	Patent expiry
Abelcet	Amphotericin B	Fungal infections	1995	2014
AmBisome	Amphotericin B	Fungal infections	1990 (EU)/ 1997(US)	2008/2016
Amphotec	Amphotericin B	Fungal infections	1996	2015
Arikayce	Amikacin	Mycobacterium avium complex lung disease	2018	2037
Caelyx (EU)/ Doxil (US)	Doxorubicin hydrochloride (PEGylated)	Breast neoplasms; multiple myeloma; ovarian neoplasms; Kaposi's sarcoma	1995	2014
DaunoXome	Daunorubicin	Cancer advanced HIV-associated Kaposi's sarcoma	1996	2015
DepoCyt	Cytarabine/Ara-C	Neoplastic meningitis	1999	2018
DepoCyt[a]	Cytarabine	Meningeal neoplasms	1999	2018
DepoDur	Morphine	Pain relief	2004	2017
Epaxal	Inactivated hepatitis A virus (strain RGSB)	Hepatitis A	1993	2012
Exparel	Bupivacaine	Anaesthetic	2011	2018
Inflexal	Inactivated haemagglutinin of Influenza virus strains A and B	Influenza	1997	2016
Marqibo	Vincristine	Philadelphia chromosome-negative acute lymphoblastic leukaemia	2012	2020
Mepact	Mifamurtide	Osteosarcoma	2009	2028
Myocet	Doxorubicin hydrochloride	Breast neoplasms	2000	2019
Nocita	Bupivacaine	Long-acting local anaesthetic	2017	2036
Onivyde	Irinotecan	Combination therapy with fluorouracil and leucovorin in metastatic adenocarcinoma of the pancreas	2015	2034
Visudyne	Verteporfin	Macular degeneration, degenerative myopia	2000	2016
Vyxeos	Daunorubicin/cytarabine	Acute myeloid leukaemia	2017(US) 2018 (EU)	2036 (US) 2037 (EU)

[a]Production stopped due to manufacturing issues.

concentration limit of 5000 ppm.[7] Considering these issues, various approaches have reported using less hazardous solvents such as isopropyl alcohol[8] and ethanol.[9]

Given these issues and the need to develop more sustainable production practices, we have investigated and developed an easy-to-adopt manufacturing method for drug-loaded liposomes that does not require the use of organic solvents (Figure 1). Liposome size control was achieved using the electric benchtop laboratory M110P Microfluidizer processor. Using this solvent-free and scalable process, we have manufactured doxorubicin-loaded liposomes (PEGylated and non-PEGylated) and amphotericin B-loaded liposomes with physicochemical attributes mapped to clinically approved products in terms of size and drug loading.

Table 2 Generic doxorubicin and amphotericin B liposomal products and their manufacturer

Drug	Original product	Generic version	Manufacturer
Doxorubicin Hydrochloride	Doxil	Adropeg 20	Axiommax Oncology Pvt. Ltd
		DOXOrubicin	Dr. Reddy's
		Doxulip	United Biotech
		i-dox	Getwell
		Lipodox	Sun Pharma
		Lippod	Celon Labs
		Natdox-LP	Natco Pharma Ltd.
		Pegadria 50	Intas Pharmaceuticals Ltd.
		Rubilong	Zuventus Healthcare Ltd.
		SinaDoxosome	Exir Nano Sina Co
Amphotericin B	AmBisome	Abhope	Abbott
		Ambilip	United Biotech
		Amflight	Celon Labs
		Amphonex	Bharat serums and vaccines Ltd.
		Phosome 10	Cipla

Materials and Methods

Materials

Egg phosphatidylcholine (Egg PC), hydrogenated soy phosphatidylcholine (HSPC), 1,2-distearoyl phosphatidylcholine (DSPC), 1,2-distearoyl-sn-glycero-3-phospho-(1′-rac-glycerol) (DSPG) and 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-

Table 3 Methods and solvent used during manufacturing of liposomes

Method	Mechanism	Solvents
Bangham method[32-34]	Rehydration of thin lipid film	Chloroform and methanol
Reverse-phase evaporation[35]	Aqueous phase added to the organic phase and evaporated to form liposomes	Diethyl ether, isopropyl ether, halothane and trifluorotrichloroethane
Detergent depletion[36]	Liposomes formed through detergent lipid interaction	Chloroform and methanol
Microfluidic channel[37,38]	In-line precipitation of liposome from the organic phase into aqueous	Ethanol, methanol and isopropyl alcohol
Dense Gas method[35]	Use of dense gas as a solvent for lipids instead of organic solvents	Ethanol and methanol
Ethanol/ether injection[39-41]	Precipitation of liposome from the organic phase into aqueous	Ethanoland diethyl ether
Supercritical fluid method[35]	Use of supercritical fluids as a solvent for lipids instead of organic solvents	Ethanol and methanol
Supercritical antisolvent method[42]	Lipids dissolve readily in $scCO_2$ and then precipitate in the form of ultrafine particles	Chloroform,methanol and ethanol
Dual asymmetric centrifugation method[35,43]	Highly concentrated lipid dispersion system diluted by a suitable aqueous phase during centrifugation	Chloroform, methanol and ethanol
Membrane contactor method[44]	The aqueous phase is poured into the module by the action of a pump, while the organic phase is placed in the pressurised vessel	Ethanol

N-[methoxy(polyethylene glycol)-2000] (DSPE-PEG 2000) were obtained from Lipoid (Ludwigshafen, Germany). Sodium succinate, cholesterol (SyntheChol), amphotericin B, 2-hydroxypropyl-cyclodextrin, citric acid, sodium citrate tribasic, L-histidine, ammonium sulfate, doxorubicin HCl European Pharmacopoeia reference standard, sodium azide (NaN_3) and ammonium bicarbonate (NH_4HCO_3) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Sucrose and sodium hydroxide were obtained from Fisher Scientific, Loughborough, UK. Dialysis tubing 300 kD and mPES 750 and 100 kD columns were purchased from Repligen Corporation, California, USA. Other chemicals were used at analytical grade, and mQ-water was provided by an in-house system.

Methods

Organic solvent-free manufacturing of doxorubicin-loaded liposomes

Empty PEGylated and non-PEGylated liposomes (HSPC : Chol : DSPE-PEG2000 56 : 38 : 5 and Egg PC : Chol 45 : 55 molar ratio) were prepared by mixing powdered lipids and cholesterol with 100 mL of hydration buffer at 70 °C as per Table 4. The lipid dispersion was high shear mixed (high shear mixer IKA T25 basic with S 25 N – 18 G dispersing tool) at 8000 rpm for 1 h at 70 °C. Liposomes were size reduced at 65–70 °C using the M110P Microfluidizer processor (Microfluidics Inc., Westwood, MA, USA). Water was circulated at a temperature above the transition temperature of the phospholipids (70 °C) in the heat exchanger to achieve the necessary fluidity of the liposomal bilayer for particle size reduction. The M110P Microfluidizer processor was primed with buffer, and samples were processed at pressures of 5000 psi, 8000 psi, 15 000 psi, 18 000 psi and 20 000 psi for up to three processing passes. Formulations were subjected to tangential flow filtration (TFF) for buffer exchange and concentration control (Figure 1). Doxorubicin was subsequently added to liposomes and incubated at 60 °C for 10 min to actively load the drug using a pH gradient.

Organic solvent-free manufacturing of amphotericin B-loaded liposomes

Succinate buffer (10 mM, pH 2) with sucrose (9% w/v) was heated at 70 °C using a water bath. DSPG (63.3 mg) was added to succinate buffer and high shear mixed for 15 min. Basic amphotericin B solution (37.6 mg) was prepared using 2 M sodium hydroxide. Amphotericin B solution was added to the acidified DSPG suspension at 70 °C and vortexed for 15 min. The DSPG-amphotericin B suspension (pH 5.5) was high shear mixed for 1 h at 70 °C with powdered HSPC (159.8 mg) and cholesterol (39.3 mg). Liposomes (batch size 100–500 mL) were size reduced at 65–70 °C using the M110P Microfluidizer processor at 25 000 psi for three passes.

Characterisation of particle size, polydispersity and zeta potential using dynamic light scattering

The particle size, measured as the hydrodynamic diameter, polydispersity index (PDI) and zeta potential were measured by dynamic light scattering using a Zetasizer Nano ZS (Malvern Panalytical Ltd, Worcestershire, UK)

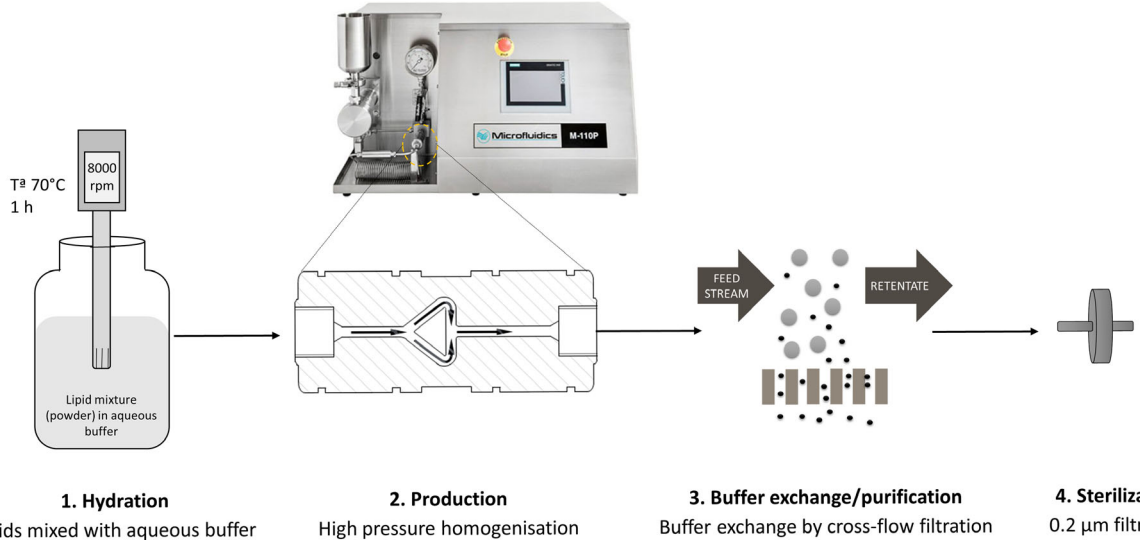

Figure 1 Schematic representation of liposome manufacture, buffer exchange/purification, drug loading and sterilisation. Liposomes were manufactured by adding powdered lipids to aqueous buffers without organic solvents (1), processed using high-pressure homogenisation (M110P Microfluidizer processor) (2), and subjected to tangential flow filtration for buffer exchange and purification (3), and finally, drug-loaded liposomes were sterile-filtered using a 0.22µm filter (4).

equipped with a 633 nm laser and a detection angle of 173°. Samples were diluted to 0.2 mg/mL lipid concentration in water, and the values of water were used for refractive index and viscosity. Zetasizer Software v.7.11 (Malvern Panalytical Ltd.) was used for the acquisition of data.

Removal of free drug and buffer exchange with tangential flow filtration

Liposomes were purified by TFF using a KrosFlo KR2i system (Waltham, MA, USA) and mPES 750 kD and 100 kD columns for amphotericin B and doxorubicin formulations, respectively. Formulations were concentrated to achieve final lipid concentrations (28, 16 and 8 mg/mL for amphotericin B, PEGylated and non-PEGylated doxorubicin-loaded liposomes, respectively) and washed for 10 diafiltration cycles per 1 mL of formulation using an appropriate buffer for buffer exchange and to remove unentrapped drug.

Quantification of drug loading

Quantification of doxorubicin was performed using a microplate reader model 680 (Bio-Rad Laboratories. Inc., Hertfordshire, UK) measuring the UV absorbance at 490 nm. Liposomes were solubilised with 50% 2-propranolol (*v/v*). Calibration curves were performed under the same conditions as the samples. The limit of detection (LOD) and quantification (LOQ) were 0.05 and 0.15 mg/mL, respectively. Quantification of amphotericin B was performed by reverse-phase high-performance liquid chromatography (HPLC) (Agilent 1100) using a UV-VIS detector connected to the instrument. A Gemini C18 column with 110 Å pore size 150 × 4.60 mm 5 µm (Phenomenex, Macclesfield, UK) was used as a stationary phase. A 1 mL/min flow rate was used with an 18 min elution gradient, composed of solvent A (0.1 % perchloric acid in water) and solvent B (100% acetonitrile) at 408 nm. Initially, the gradient increased from 95 : 5 (A : B) to 5 : 95 (A : B) at 5 min, before returning to an initial composition of 95 : 5 (A : B) at 15 min which was maintained for 2 min until the end of the analysis. The sample injection volume was 20 µL. LOD and LOQ were 0.09 and 0.28 µg/mL, respectively.

Morphological characterisation of liposomes via CryoTEM

Samples for microscopy were prepared by placing 5 µL of liposomes onto a 400-mesh lacey carbon-coated grid using single-sided blotting for 2 s, then immediately immersing the sample grid into nitrogen-cooled ethane (100% ethane). Liposome morphology was then observed using the Jeol Jem F-200 microscope (Joel, Tokyo, Japan) at liquid nitrogen temperature and 200 kV.

Drug release studies using USP-4 dissolution apparatus

The effect of manufacturing conditions, including pressure and number of passes, on doxorubicin release was studied

Table 4 Doxorubicin-loaded formulation composition used during manufacturing

Composition	PEGylated formulation	Non-PEGylated formulation
HSPC	4.8 mg/mL	–
EggPC	–	3.9 mg/mL
DSPE-PEG 2000	1.6 mg/mL	–
Cholesterol	1.6 mg/mL	1.6 mg/mL
Doxorubicin	2 mg/mL	2 mg/mL
Hydration buffer	250 mM Ammonium sulfate pH 5.5	300 mM Citrate buffer pH 5.5
External buffer	Sucrose-Histidine pH 6.5	Sodium carbonate pH 7.3

using USP-4 flow-through CE7-smart (SOTAX) apparatus using a method previously developed by Yuan *et al.*[10] which was shown to offer discriminatory assessment of liposomal drug release from these formulations. Briefly, 0.8 mL of 2 mg/mL free doxorubicin (in 10% *w/v* sucrose and 10 mM histidine, pH 6.5) or doxorubicin-loaded liposomes was placed in 300 kD dialysis tubes and inserted into USP-4 flow-through cells with 39.2 mL 100 mM NH_4HCO_3, 75 mM 2-(N-morpholino) ethane sulfonic acid (MES), 5% *w/v* hydroxypropyl-cyclodextrin (HP-CD), 5% *w/v* sucrose and 0.02% *w/v* NaN_3 (pH 6) as a release medium (the total volume of release media was 40 mL, and the final doxorubicin concentration in the release media was equivalent to 20 μg/mL). The flow rate and running temperature of the release medium were set at 16 mL/min and 45 °C, respectively.[10] The cumulative release was calculated 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).[10]

Results

Size-controlled high drug-loaded liposomes can be produced by the solvent-free method

To investigate the effect of pressure and number of homogenisation passes during manufacturing, PEGylated liposomes (as per Table 4) were prepared using the organic solvent-free technique at an 8 mg/mL lipid concentration. After initial hydration of the lipids and production of multilamellar vesicles, liposomes had a z-average diameter of approximately 1500 nm and a D90 of approximately 2600 nm (Figure 2). Liposomes were then down-sized at 15 000, 18 000 and 20 000 psi with up to three homogenisation passes using the M110P Microfluidizer processor. Figure 2 shows that the pressure and number of passes adopted during manufacturing significantly control liposome size and PDI. Formulations manufactured at 15 000 psi significantly reduced ($P < 0.05$) the *z*-average diameter to approx. 100 nm at pass 3 with a PDI of 0.29. The D50 size was similar to the *z*-average, with the D90 being approximately 200 nm (Figure 2). To enhance size reduction, higher pressures were adopted (Figure 2); at 18 000 psi, liposomes significantly decreased in size ($P < 0.05$) to 120 nm after 1 pass, down to 95 nm (*z*-average diameter) by pass 3 (0.19 PDI). The D10, D50 and D90 also reduced in a similar pattern (Figure 2). Further increasing the pressure to 20 000 psi produced the most rapid size reduction ($P < 0.05$) and the smallest particles with only two passes being required to achieve <100 nm size and PDI < 0.2 (Figure 2). Across all formulations, the zeta potential was in the range of 5 to −5 mV, with no significant impact from the amount of pressure used. In general, the pressure was the parameter affecting the physicochemical characteristics the most, with all three pressures giving values significantly different from each other ($P < 0.05$). After the first pass, the number of passes did not significantly affect the vesicle size. However, the main vesicle characteristic affected here was the PDI ($P < 0.05$). After the second pass, the PDI reached a plateau of around 0.2 for pressures 18 000 and 20 000 psi (Figure 2).

To further explore this, PEGylated liposome formulations were manufactured at 18 000 and 20 000 psi, and a pH gradient established by exchanging the external buffer from 250 mM ammonium sulfate (pH 5.5) to sucrose-histidine using TFF. Doxorubicin was then loaded into these formulations, and finally, the formulations were sterilised (0.22 μm filtration) (Figure 3). Particle size (*z*-average diameter) after production at 18 000 psi was approx. 105 nm after two passes, dropping to 95 nm after three passes. At 20 000 psi, the sizes dropped by approximately 10 nm to 95 and 85 nm after passes 2 and 3, respectively (Figure 3). Across all four test parameters, the PDI of the liposomes remained low (<0.2) demonstrating a high level of homogeneity. With these formulations, which were still in ammonium sulfate buffer and without drug-loaded, the zeta potential is neutral (Figure 3). Liposomes were then subjected to buffer exchange (using histidine-sucrose buffer) to set up the pH gradient. This did not significantly change the particle size of the various liposomes but did significantly ($P < 0.05$) reduce the zeta potential to −35 - −40 mV for all four preparations tested (Figure 3). Using the established pH gradient, liposomes were actively loaded with doxorubicin. Drug loading was >90%, irrespective of the pressure or number of passes used for the formulations (Figure 3), while subsequent sterilisation had no significant effect on these attributes.

Liposomes prepared by the organic solvent-free method were compared to liposomes produced via the traditional

	Pass	Particle size (nm)	D10	D50	D90	PDI	Zeta Potential (mV)	Intensity peaks
15 000 PSI	0*	1466 ± 155	112 ± 13	1310 ± 615	2570 ± 2120	0.96 ± 0.05	4.7 ± 3.2	
	1	132 ± 2	64 ± 2	123 ± 4	405 ± 14	0.42 ± 0.01	−1.0 ± 0.6	
	2	111 ± 1	61 ± 1	112 ± 2	537 ± 56	0.32 ± 0.03	−3.2 ± 1.2	
	3	101 ± 2	55 ± 1	100 ± 3	194 ± 18	0.29 ± 0.04	−5.7 ± 0.7	
18 000 PSI	1	120 ± 1	65 ± 2	131 ± 4	338 ± 76	0.25 ± 0.01	−0.4 ± 1.2	
	2	103 ± 2	58 ± 2	114 ± 5	242 ± 20	0.21 ± 0.01	−2.1 ± 1.2	
	3	95 ± 2	56 ± 3	100 ± 2	189 ± 4	0.19 ± 0.01	−1.5 ± 0.8	
20 000 PSI	1	106 ± 2	61 ± 1	110 ± 8	222 ± 34	0.26 ± 0.02	−1.4 ± 0.3	
	2	96 ± 1	54 ± 2	104 ± 1	217 ± 21	0.22 ± 0.01	−3.8 ± 0.5	
	3	85 ± 2	53 ± 1	88 ± 3	154 ± 12	0.18 ± 0.01	−5.1 ± 2.7	

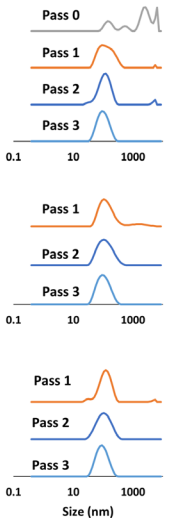

Figure 2 Effect of pressure and number of passes on PEGylated formulation. Particle size (*z*-average diameter, nm), *D*-value intercepts for 10%, 50% and 90% of the cumulative mass (D10, D50, D90; based on intensity), PDI and zeta potential (mV) were measured during processing using the M110P Microfluidizer processor. Empty HSPC : Chol : DSPE-PEG2000 liposomes were manufactured as outlined in Table 4 at8 mg/mL lipid concentration using 15 000, 18 000 and 20 000 psi for three passes. Results represent mean ± SD from three independent batches.

lipid hydration and sonication method (Figure 4). These liposomes were prepared using either HSPC or DSPC as the base lipid. With the lipid hydration method, the *z*-average diameter was around 100–120 nm, with low PDI (<0.2) and high drug loading (>90%). CryoTEM images show classical oval-/ellipsoidal-shaped liposomes as a result of the formation of long nanocrystals of doxorubicin-sulfate within the aqueous phase. When these formulations were prepared by the solvent-free method, comparable liposome morphology can be seen irrespective of the base lipid used (Figure 4).

Pressure and number of passes influence liposome particle size but not doxorubicin release

To further investigate the impact of manufacturing attributes, doxorubicin-loaded PEGylated liposomes were prepared and subjected to drug release testing using the USP-4 dissolution apparatus with a protocol previously developed to discriminate between doxorubicin-loaded liposomes.[10] From the results in Figure 5a, we can see that there were no significant differences in doxorubicin release profiles of liposomes prepared after two or three passes at 18 000 or 20 000 psi, with approximately 60% doxorubicin release after 6 h. On the other hand, free doxorubicin showed 100% release after 2 h (Figure 5a). Additionally, we can see that the PDI was unchanged after drug release, with 0.2 PDI irrespective of the down-sizing pressure (Figure 5b). However, particle sizes significantly ($P < 0.05$) decreased after drug release, possibly as a result of the loss of the drug nanocrystals within the liposomes.

Solvent-free production can also be applied to non-PEGylated liposomes

To further investigate whether doxorubicin-loaded non-PEGylated liposomes (Egg PC : Cholesterol) can be manufactured using the solvent-free manufacturing technique, empty formulations (as per Table 4) were prepared at 5000, 8000, 10 000, 15 000 and 18 000 psi. Once again, the *z*-average diameter, PDI and zeta potential were measured at each pass. By increasing the pressure during manufacturing, liposome size significantly ($P < 0.05$) decreased from 287 to 137 nm (pass 1 to 3; 5000 psi), from 217 to 117 nm (8000 psi), from 126 to 103 nm (10 000 psi), from 110 to 89 nm (15 000 psi) and from 103 to 78 nm (18 000 psi) (Figure 6a). Regarding PDI, formulations manufactured above 10 000 psi pressure produced homogeneous suspensions with PDI commonly below 0.2 at pass 3 (Figure 6a). Across all the formulations, the zeta potential remained neutral, with values between −5 and 5 mV (Figure 6b).

Based on these results, non-PEGylated liposomes were characterised after production at 18 000 psi, subjected to buffer exchange using TFF and loaded with doxorubicin. The formulations were sterilised via 0.22 µm filtration

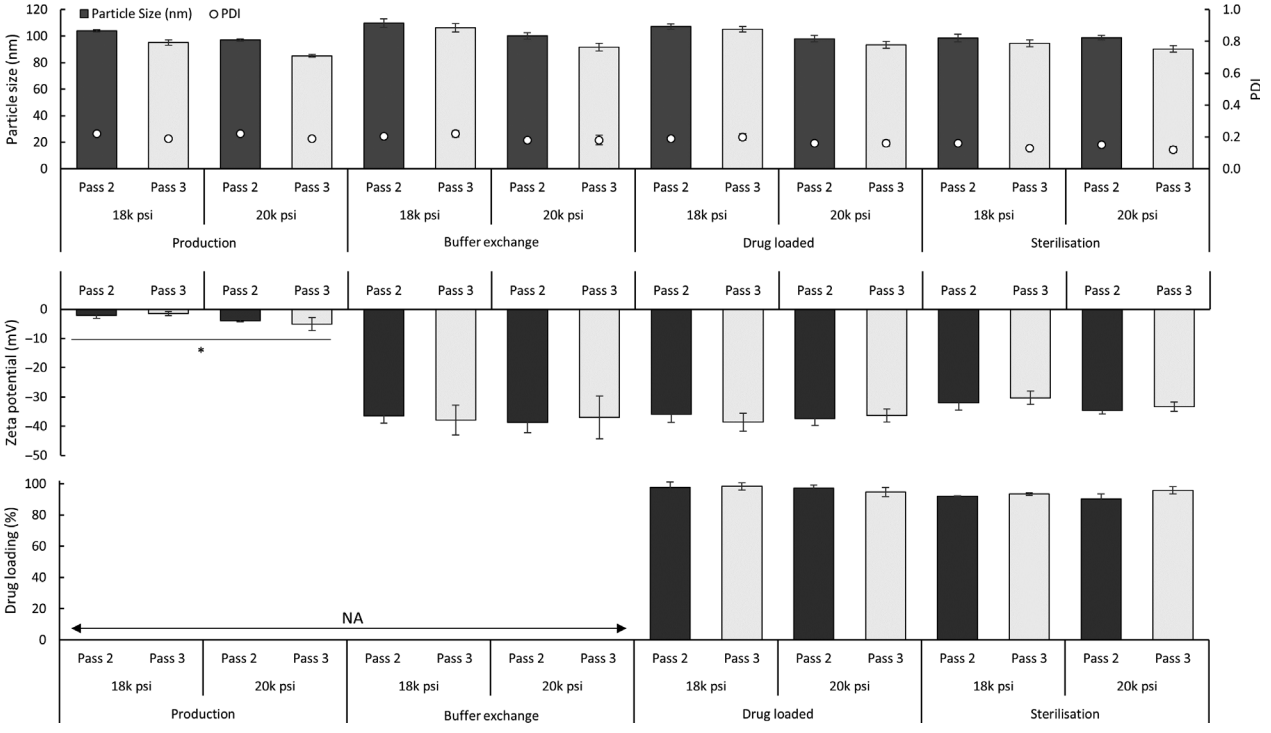

Figure 3 Effect of pressure and number of passes on the PEGylated formulation: Particle size, PDI and drug loading. Liposomes were manufactured as outlined in Table 4 at 18 000 and 20 000 psi for two or three passes. Formulations were subjected to tangential flow filtration for buffer exchange, followed by the addition of doxorubicin to load into the liposomes. Doxorubicin-loaded liposomes were sterile-filtered using 0.22μm filters. Results are shown as (a) particle size (*z*-average diameter, nm) and PDI (columns and open circles, respectively), (b) zeta potential (mV) and (c) % drug loading of doxorubicin. Results represent mean ± SD from three independent batches.

(Figure 7). After three microfluidic passes, purification and sterilisation, liposomes were 90–100 nm in size, unimodal in nature (PDI values < 0.2) and with high drug loading (>75%) (Figure 7a–c). With this formulation, liposomes encapsulate doxorubicin-citrate rather than doxorubicin-sulfate similar to the marketed formulation Myocet. This results in a different structural morphology (Figure 7d) with CryoTEM images of doxorubicin fibre bundles having straight, curved and circular geometries due to the fibres being more flexible than doxorubicin-sulfate. These results show that by using the organic solvent-free method, we can manufacture doxorubicin-citrate-loaded, non-PEGylated liposomes with physicochemical attributes comparable to Myocet (Figure 7).

Solvent-free production can also be adopted to manufacture amphotericin B-loaded liposomes

The applicability of the organic solvent-free technique to manufacture liposomes with a water-insoluble drug was also demonstrated by preparing amphotericin B-loaded liposomes. For this formulation, a pressure of 25 000 psi was employed (based on pilot data; data not shown) with up to three passes of homogenisation applied using M110P Microfluidizer processor (Figure 8a). During size reduction, the anionic zeta potential of the liposomes remained between −40 and −50 mV (Figure 8b) and the liposomes became unimodal in nature (Figure 8c). Again, manufacturing this formulation with M110P Microfluidizer processor showed high (98–100%) drug loading throughout the homogenisation process (Figure 8d). Using this manufacturing process, we were able to produce amphotericin B-loaded liposomes of 100–110 nm particle size, with a 0.2 PDI (Figure 8e) which are generally single or bilamellar in nature (Figure 8f).

Liposome composition influences size reduction capability

To further consider the impact of lipid choice and lipid concentration, formulations containing low transition lipids (Egg PC) and high transition lipids (HSPC), with or without DSPE-PEG2000 and DSPG, were prepared at higher concentrations to determine the highest manufacturing concentration limits using the organic solvent-free

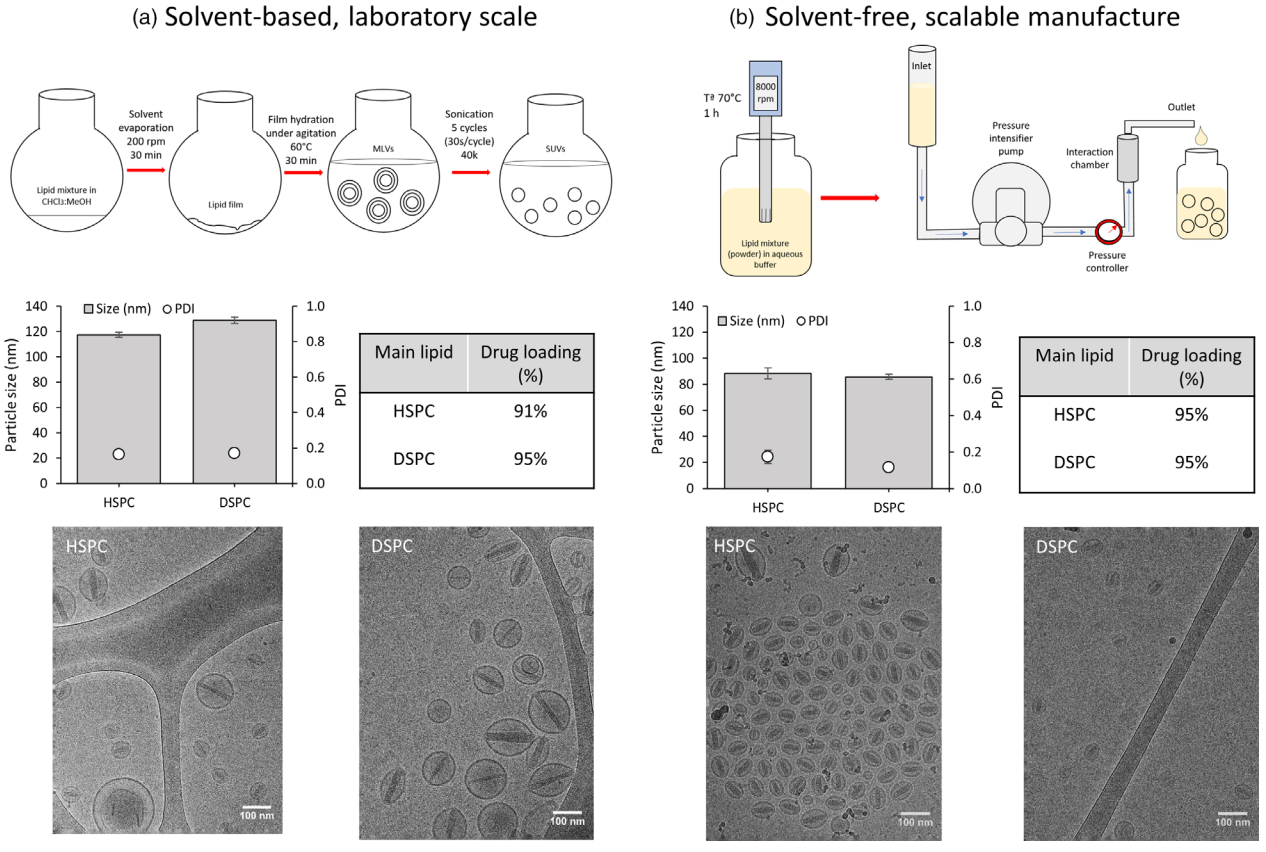

Figure 4 The physicochemical characteristics and morphology of PEGylated doxorubicin-loaded liposomes. HSPC and DSPC liposomes with doxorubicin loaded via a pH gradient (intravesicular salt solution: 250 mM ammonium sulfate, pH 5.5; extraliposomal buffer: sucrose-histidine pH 6.5) were prepared by either conventional lipid thin film hydration followed by sonication (solvent-based, laboratory scale) or with the organic solvent-free method (solvent-free, scalable manufacture).

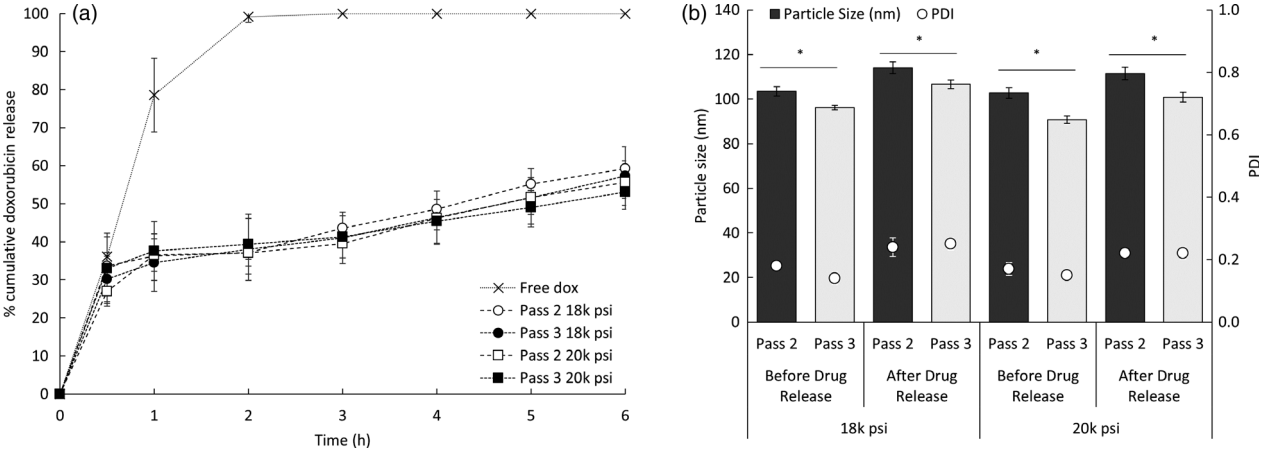

Figure 5 Doxorubicin release profiles of PEGylated doxorubicin-loaded liposomes using flow-through USP-4 apparatus. Final doxorubicin concentration in the release media was equivalent to 20 μg/mL, and doxorubicin release profile was investigated over 6 h at 45 °C. Results are shown as (a) effect of pressure and number of passes during manufacturing on doxorubicin release and (b) particle size (*z*-average diameter, nm) and PDI (columns and open circles, respectively) before and after doxorubicin release. Results represent mean ± SD from three independent batches.

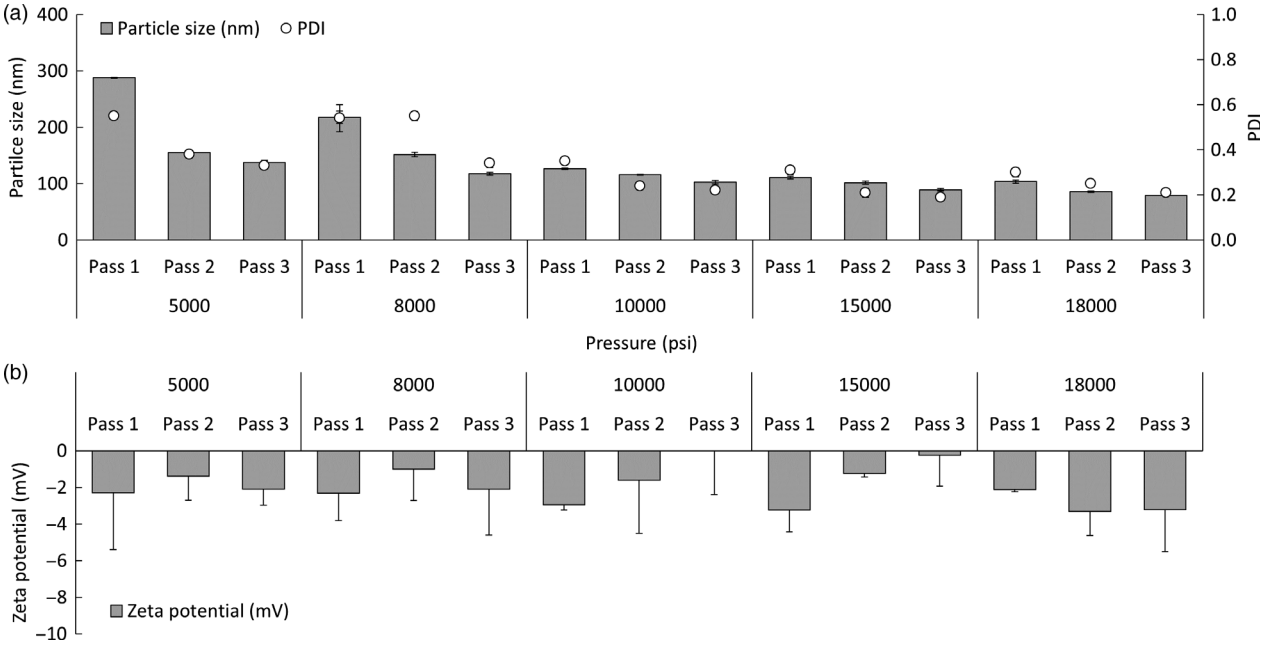

Figure 6 Effect of pressure and number of passes on particle size, PDI and drug loading of the non-PEGylated liposome formulation. Empty non-PEGylated liposomes were manufactured as outlined in Table 4 at 5000, 8000, 10 000, 15 000 and 18 000 psi for three passes. Results are shown as (a) particle size (*z*-average diameter, nm) and PDI (columns and open circles, respectively) and (b) zeta potential (mV). Results represent mean ± SD from three independent batches.

method (Figure 9). Our results suggest that Egg PC-based liposomes can be easily prepared at concentrations up to 60 mg/mL total lipid with PDI values of <0.2 being achieved at 3–5 processing passes (Figure 9a–c). With HPSC-based formulations (Figure 9d–f), the addition of DSPG or DSPE-PEG2000 tended to allow higher concentrations of lipids to be used compared with HSPC : Chol formulations (40 mg/mL for HSPC : Chol (Figure 9d) compared with 80 mg/mL for formulations containing DSPE-PEG2000 or DSPG; Figure 9e,f).

Discussion

There are a range of conventional methods available to manufacture liposomal formulations, for example, the lipid hydration method, detergent depletion, ether/ethanol injection, reverse-phase evaporation and emulsion methods.[5,11] It should be noted that the majority of conventional liposome preparation procedures involve the use of organic solvents (Table 3). When we consider larger scale production of liposomes, and nanomedicines in general, the use of solvents and issues with scalable production methods present challenges. Indeed, manufacturing issues can lead to supply shortage and eventually stoppage asseen with the Doxil/Caelyx product.[12]

Here, we demonstrate an organic solvent-free method to produce a selection of drug-loaded liposomes based on clinically approved products including those incorporated within the aqueous core (Doxil and Myocet) and those where the drug is incorporated within the bilayer (AmBisome). In all three formulations, the drug is loaded within the liposomes to improve targeting of the drug and reduce off-target toxicity. For Doxil (PEGylated liposomes) and Myocet (non-PEGylated liposomes), the drug is loaded after liposome production via remote loading[13] and formulations mapped to both of these marketed formulations can be produced without solvent (Figures 4 and 7, respectively). When doxorubicin is remotely loaded into PEGylated liposomes via a transmembrane ammonium sulfate gradient, long nanocrystals of doxorubicin-sulfate are formed in the aqueous phase generating ellipsoidal liposomes[14] as confirmed in Figure 4. With AmBisome, the drug is incorporated within the bilayer during production. Amphotericin B is very poorly soluble in aqueous media[15] and highly soluble in hydrophobic media such as lipid membrane bilayers.[16] An increase in solubility and reduction of toxicity can be achieved by conversion of oligomers of amphotericin B to hetero-aggregates containing detergents or lipids,[15] including liposomal bilayers.[17] Liposomal amphotericin B formulations are thus less toxic than conventional formulations and more effective in treating fungal infections allowing for higher exposure and longer duration of therapy.[18–20] Currently, manufacturing steps of AmBisome® involve solubilisation of lipids in an organic

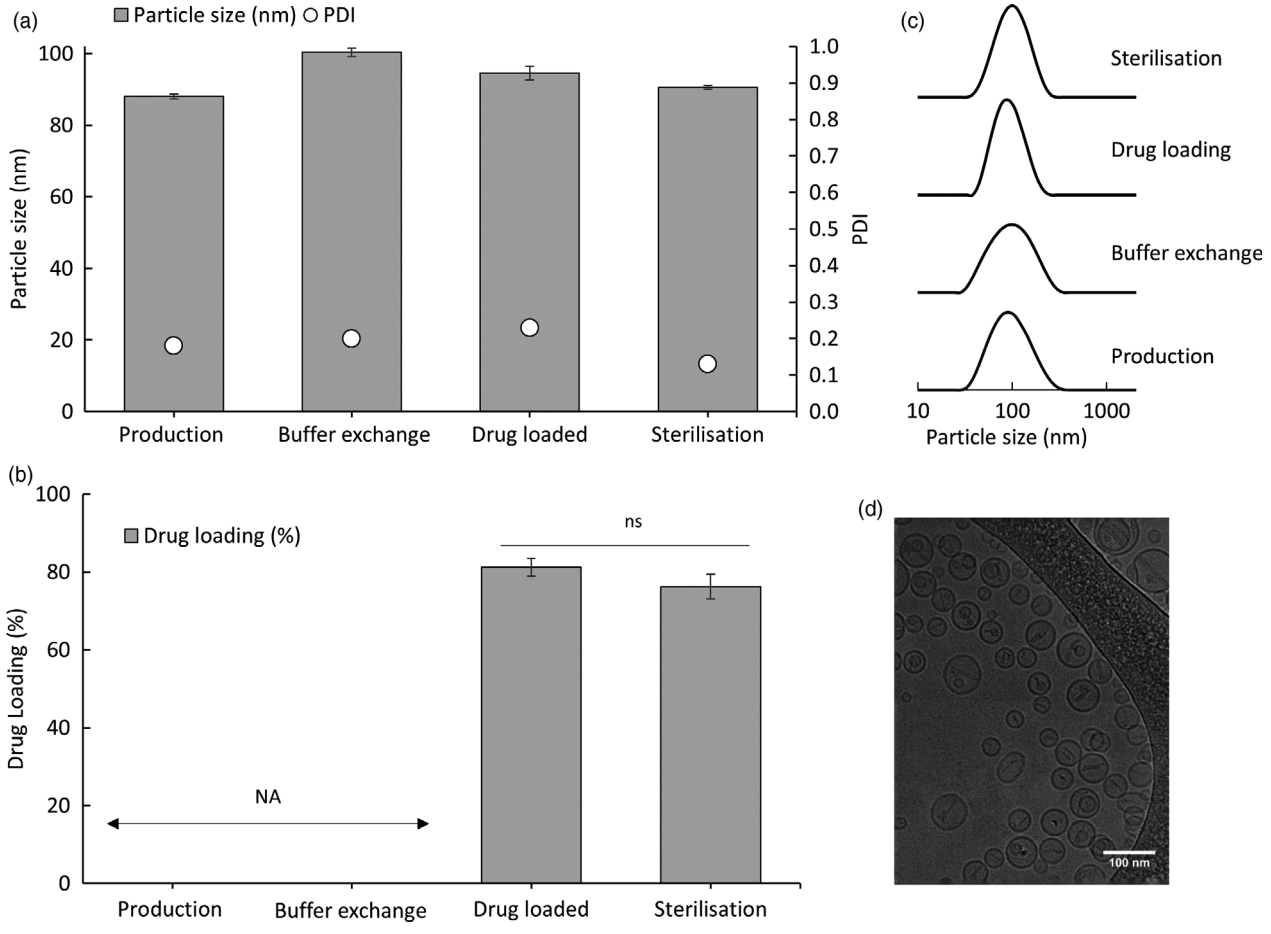

Figure 7 In-process manufacturing characterisation of the doxorubicin-loaded non-PEGylated formulation. Liposomes were manufactured as outlined in Table 4 at 20 000 psi for three passes. Formulations were subjected to tangential flow filtration for buffer exchange followed by the addition of doxorubicin to load into the liposomes. Doxorubicin-loaded liposomes were sterile-filtered using a 0.22μm filter. Results are shown as (a) particle size (*z*-average diameter, nm) and PDI (columns and open circles, respectively), (b) % drug loading of doxorubicin and (c) particle size intensity plots. CryoTEM images of the liposomes are shown in (d). Results represent mean ± SD from three independent batches

solvent such as chloroform and methanol, acidification of lipids and drug complex, removal of solvent and hydration to form liposomes followed by size reduction.[21,22] Figure 8 demonstrates that amphotericin B-loaded liposomes can be manufactured by an organic solvent-free technique using the M110P Microfluidizer processor. In this manufacturing process, the amphotericin B and DSPG complex remains an essential element.[23] Since amphotericin B is water-insoluble, it is difficult to make a complex in an aqueous phase. Therefore, the solubility of amphotericin B was altered using the protonation of the amine group from amphotericin B. Acidified DSPG receives the proton from amphotericin B and forms a complex in aqueous phases.

Using the developed organic solvent-free method, we are able to produce liposome products that map to the physicochemical specifications of each of these marketed products at high throughputs. When using the high-pressure Microfluidizer technology, we are able to produce liposomes from bench-scale to high throughput. Size reduction of the liposomes is achieved by the layers of liposomes being stripped away during homogenisation. This results in liposomal formulations with a narrow size distribution. With this process, selection of pressure and number of passes during manufacturing are both critical process parameters as both impact on particle size and PDI. However, within our studies, we see no evidence of impact on zeta potential, drug loading and drug release. Therefore, we demonstrate that liposomal formulations can be manufactured and particle size controlled through a range of pressures and number of homogenisation passes.

Studying the release profile of generic liposome formulations, such as the doxorubicin formulations, is important for quality control and comparability studies.[10] To examine drug release from the liposomal formulations, we used

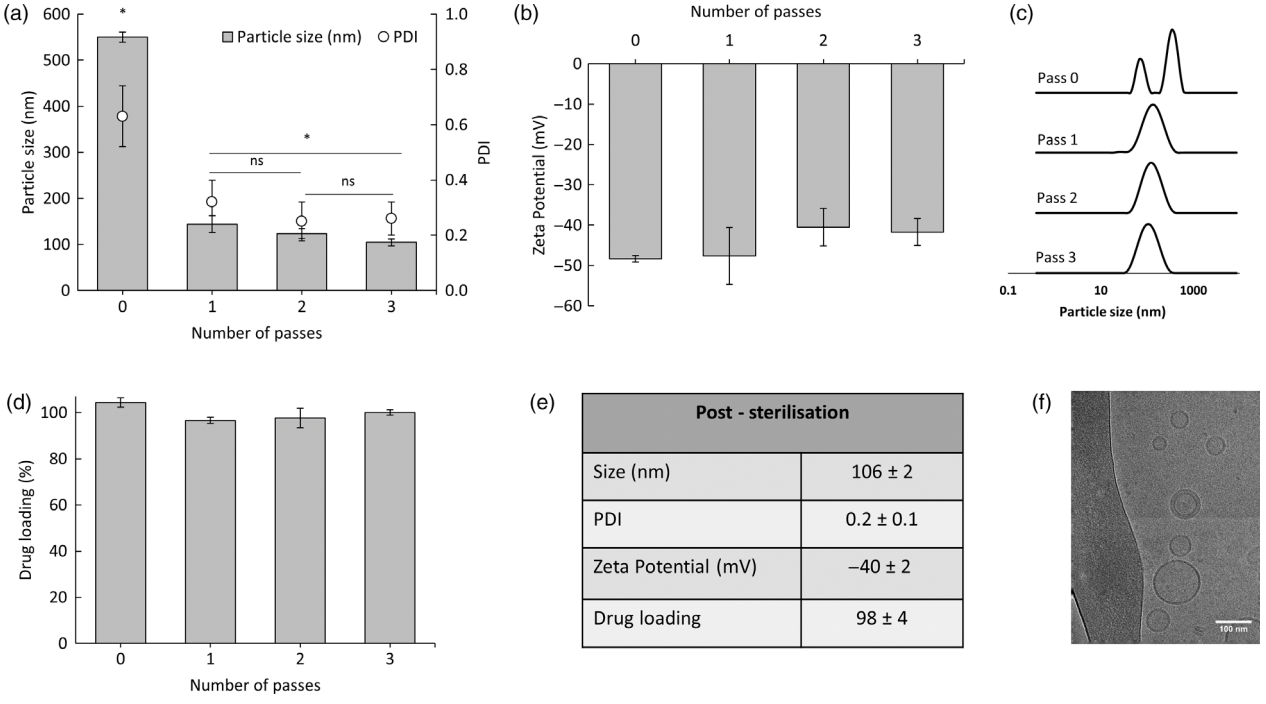

Figure 8 Formulation characterisation of the amphotericin B-loaded formulation. Liposomes were manufactured at 25 000 psi for three number of passes. Formulations were subjected to tangential flow filtration to remove unentrapped amphotericin B and were sterile-filtered using a 0.22μm filter. Results are shown as (a) particle size (nm) and PDI (columns and open circles, respectively), (b) zeta potential (mV), (c) particle size intensity plots, (d) % drug loading of amphotericin B and (e) post-sterilisation formulation characteristics. CryoTEM images of the liposomes are shown in (f). Results represent mean ± SD from three independent batches

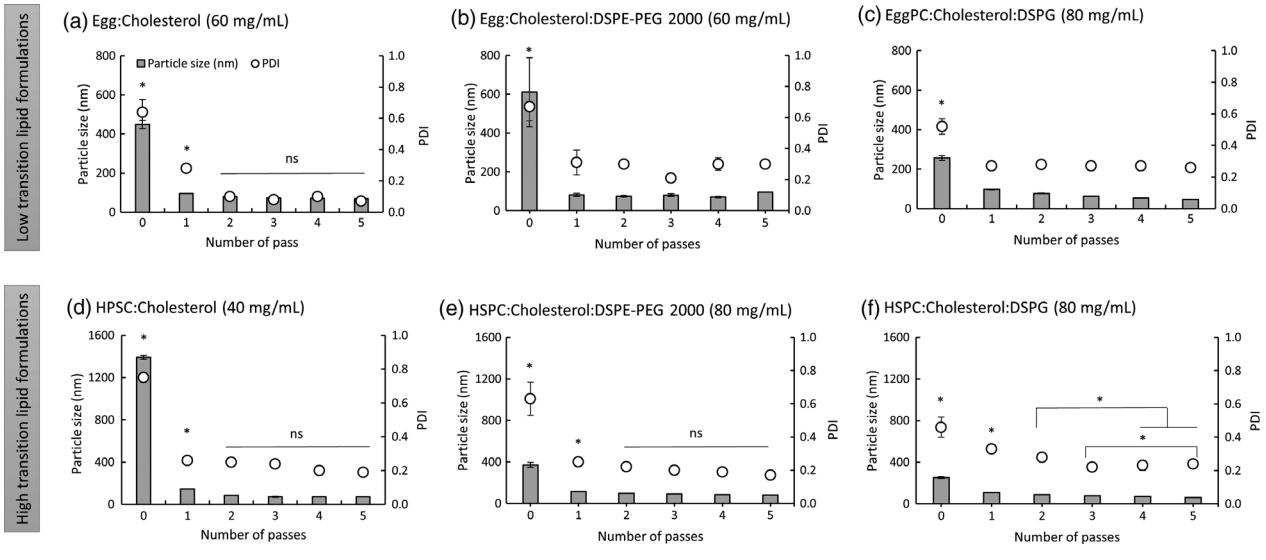

Figure 9 The impact of lipid choice on manufacturing concentration limits (1–8% *w/v* lipids to buffer). Liposomes were prepared at 20 000 psi at high concentrations and particle size (nm) and PDI (columns and open circles, respectively) measured. (a) Egg PC : Cholesterol (3 : 1 *w/w* (60 mg/mL), (b) Egg PC : Cholesterol : DSPE-PEG 2000 (3 : 1 : 1 *w/w*; 60 mg/mL), (c) Egg PC : Cholesterol : DSPG (3 : 1 : 1 *w/w*; 80 mg/mL), (d) HSPC : Cholesterol (3 : 1 *w/w*; 40 mg/mL), (e) HSPC : Cholesterol : DSPE-PEG 2000 (3 : 1 : 1 *w/w*; 80 mg/mL) and (f) HSPC : Cholesterol : DSPG (3 : 1 : 1 *w/w*; 80 mg/mL)

a dialysis insert set-up of USP-4. From Figure 5, we can see that over 50% of doxorubicin released from liposomes in 6 h (as previously shown ref.[10]). Furthermore, the doxorubicin release pattern was not affected by liposome production at different pressures nor the number of passes, indicating the performance of liposomes was not affected due to the selected manufacturing parameters which can be adopted as normal operational parameter settings.

When considering the impact of lipid choice on manufacturing concentration limits, not only bilayer rigidity and transition temperature,[24] but also hydrophilic chains and charge of lipids play an important factor. Results from Figure 9 demonstrated that in the presence of PEG chains or anionic lipids, at higher lipid concentrations, smaller particle sizes were observed. This may be due to the tendency for PEG to be packed at highly curved surfaces towards the aqueous phase because of its large head group which promotes the formation of smaller liposomes.[25] Also, PEG provides a strong inter-bilayer repulsion on the surface of liposomes.[26,27] These act to stabilise liposome preparations by overcoming the attractive Van der Waals forces and thus avoid aggregation. Since the attachment of PEG molecules to the surface of liposomes or anionic charge from DSPG strongly reduces the attractive forces (Van der Waals), and increases the repulsive forces (steric, electrostatic and hydration),[28,29] addition of PEG or DSPG causes disaggregation of liposome assemblies and a reduction of liposome size.

Conclusions

From our studies, we demonstrate organic solvent-free manufacturing of various liposome formulations including liposomes loaded with either doxorubicin or amphotericin B. Using the M110P Microfluidizer processor, we were able to replicate liposome physicochemical attributes of products currently approved for clinical use. Using this process, down-sizing of liposomes can be run at a flow rate of up to 120 mL/min with guaranteed scalability. Critical process parameters that controlled particle size and polydispersity were the operating pressure and number of passes. However, these did not impact drug loading (either within the aqueous core or the lipid bilayer), nor drug release when tested. This allows us to manipulate the running pressure and pass number to produce liposomes within a given target size range. Lipid concentrations of up to 80 mg/mL were processed using this manufacturing method, with liposome composition being a factor to consider when identifying optimal concentration ranges.

Declarations

Conflict of interest

The authors declare the following financial interests/personal relationships, which may be considered as potential competing interests: Yang Su is an employee of Microfluidics International Corporation.

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Authors' contribution

Swapnil Khadke, Carla B. Roces, Rachel Donaghey, Valeria Giacobbo, Yang Su and Yvonne Perrie contributed to methodology. **Swapnil Khadke, Carla B. Roces, Rachel Donaghey, Valeria Giacobbo and Yvonne Perrie** involved in formal analysis and investigated the study. **Swapnil Khadke, Carla B. Roces, Rachel Donaghey, Valeria Giacobbo, Yang Su and Yvonne Perrie** wrote, reviewed and edited the manuscript. **Swapnil Khadke, Carla B. Roces and Yvonne Perrie** visualized the data. **Yang Su and Yvonne Perrie** conceived the study. **Yvonne Perrie** supervised the study, administrated the project and acquired funding.

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