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Liposomes produced by microfluidics and extrusion: A comparison for scale-up purposes

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Abstract

Successful liposomal formulations in the clinic are severely limited due to poor translational capability of the traditional bench techniques to clinical production settings. The gold standard for liposome bench manufacturing is a multi-step and parameter dependent extrusion method. Moreover, these parameters need re-optimization for clinical production. The microfluidics technique utilizes vigorous mixing of fluids at a nanoliter scale to produce liposomes in batches from milliliters to a couple liters. The fine control of process parameters results in improved reproducibility between batches. It is inherently scalable; however, the characteristics of liposomes produced by microfluidics both in vitro and in vivo have never been compared to those produced using extrusion. In this manuscript, we describe the comparison between the traditional extrusion method to microfluidics, the new paradigm in liposome production and scale-up.

Key words: Microfluidics; Nanoassemblr; Extrusion; Liposomes; Vinblastine-N-oxide

Liposomes are phospholipid vesicles which have been utilized as a drug delivery system since the 1960s.[1,2] Structurally, they consist of a lipid bilayer and core structure which is hydrophilic in nature.[1] The presence of dual polarity in the lipid bilayer and the core makes this platform unique for encapsulation of a range of active molecules, such as anticancer drugs, peptides, hormones, proteins and antimicrobials.[1] The liposomal platform is biocompatible and has manifold applications, ranging from lengthening shelf stability to increasing retention time in the body.[2]

In spite of vast research in the translation of liposomes for clinical applications, there are only fifteen liposome-based medicines currently approved for clinical usage or in clinical trials.[3] Manufacturing of liposomes is precise and complex considering the multiple steps involved, all of which have a crucial impact on the final size, stability and functionality of the finished product.[4–6] For example, Marqibo®, vincristine sulphate liposomes, is prepared by a multi-step procedure, which includes, a) preparation of multilamellar vesicles (MLV) by lipid film hydration, b) extrusion of the MLV to unilamellar vesicles (LUV) through a polycarbonate membrane, c) establishment of a pH gradient between the internal and external buffer solution of the liposomes (sephadex PD-10 columns), and d) active loading of vincristine in the empty liposome.[7] The final formulation is assessed for size, dispersity, lamellarity, loading efficiency, safety, toxicity and pharmacokinetics, all of which are key attributes that are dependent on the method of manufacture.

The conventional methods for manufacturing liposomes usually involve rehydration of the lipid film by organic solvent injection, or mechanical methods, such as extrusion or sonication,

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for size reduction.[4] These methods are tedious and inefficient, as they result in non-uniform liposomes in terms of size, lamellarity, and polydispersity index (PDI).[8] Research has shown that the efficiency of all size reduction techniques are in the order of extrusion (103.3 ± 13.5 nm, PDI 0.2) < freeze–thaw sonication (124.7 ± 0.7 nm, PDI 0.4) < ultrasonication (235.8 ± 8.1 nm, PDI 0.4) < sonication (273.5 ± 15.3 nm, PDI 0.4) < homogenization (279.1 ± 15.8 nm, PDI 0.3).[8] Moreover, research in the lipid field highlights that both efficacy and toxicity of liposomes are affected as a function of size and PDI.[9,10] It was observed that particles less than 50 nm accumulated better in the stromal rich tumors than 100 nm sized liposomes.[10,11] However, liposomes greater than 200 nm caused complement system activation resulting in severe toxicity.[10] The pharmacokinetic circulation half-life is also governed by the size of the liposomes.[12] The general trend is the smaller the particle, the longer the circulation time. Research has shown that circulation time for liposomes decreases as size increases, such that 100 nm (8-10 h) > 220 nm (4-6 h) > 400 nm (1-4 h) for most of the conventional liposomes.[10,13–15] The numerical value of PDI ranges from 0.0 for a perfect uniform sample with respect to the particle size to 1.0 for a highly polydisperse sample with multiple particle size populations. For liposomes, values up to 0.3 are deemed acceptable; however, though the FDA's guidance for industry for liposomal products mentions size and size distribution as critical quality attributes, it does not mention the criteria for an acceptable PDI.[16]

The substantial impact of each manufacturing parameter on the final liposomal product led to the development of microfluidics, a precise, scalable, nano-precipitation method that produces liposomes with regulated size, lamellarity, and reproducible batches.[17–19] Briefly, it is a technology which involves vigorous mixing of an organic phase, usually containing lipids, with an aqueous phase on a chip by dividing liquid streams through grooves within the channels. As the organic phase in the lipid stream diffuses and dilutes into the water stream, the lipids assemble into liposomes. Stable liposomes are eventually formed when the mixture reaches equilibrium. For drug delivery applications, the set-up enables controlled, bottom-up, molecular self-assembly of liposomes *via* a mixing cartridge that allows millisecond mixing of aqueous and organic solvents. Conversely, conventional liposomal preparation consists of a multi-step process where each step dictates the final liposomal characteristics. Liposomal preparation steps include preparation and hydration of a thin film followed by a size reduction method, commonly extrusion, and purification. Overall, microfluidics is a highly precise, controlled platform which offers reduced production time, amount of drug and polymers/lipids, and provides an efficient, reproducible method for scale-up.

We have performed extensive development and optimization work for loading liposomes using extrusion, the gold standard for conventional liposomes.[20,21] However, while moving the formulation towards clinical development, we faced multiple challenges in the scale-up of liposomes prepared through the extrusion method. Hurdles in translation from bench to clinic led us to investigate liposomal development using the state-of-the-art microfluidics platform. Since the final characteristics, such as size and PDI, along with reproducibility, are the key attributes to *in vivo* liposome performance, it becomes crucial to compare liposomes prepared by extrusion to those prepared by microfluidics. In this paper, for the first time, we will compare sphingomyelin/cholesterol (spm/chol) liposomes prepared using extrusion to liposomes produced using a microfluidics platform. The liposomes encapsulate weakly basic, water-soluble vinblastine-N-oxide (CPD100), a hypoxia-activated pro-drug of vinblastine.[20] This paper focuses on two main aspects: 1. preparation and optimization of the CPD100-loaded liposomes using microfluidics, and 2. comparison of the characteristics of liposomes prepared using microfluidics to those formed using extrusion. Throughout the manuscript, we use "CPD100Li-E" for CPD100 liposomes prepared using extrusion and "CPD100Li-M" for liposomes prepared with microfluidics.

Materials

Vinblastine-N-oxide, (CPD100), is obtained from Cascade Prodrug Inc. (Eugene, OR). Egg sphingomyelin (spm) is procured from NOF America Corporation (White Plains, NY). Cholesterol (chol) and A23187 are purchased from Alfa Aesar (Haverhill, MA). Polydimethylsiloxane (PDMS) cartridges are obtained from Precision Nanosystems (BC, Canada). Supplies for cell culture including Roswell Park Memorial Institute (RPMI) 1640 medium, fetal bovine serum, trypsin ethylene-diamine-tetra acetic acid (EDTA), penicillin/streptomycin, Dulbecco's phosphate buffered saline (DPBS) and PD-10 sephadex columns for liposomal purification are acquired from VWR (Radnor, PA). ES2 Human Ovarian Clear Cell Carcinoma cell line, a distinct histopathologic subtype of epithelial ovarian cancer, is purchased from American Type Culture Collection (Manassas, VA). Cell culture reagents and disposables are procured from VWR (Radnor, PA) and Thermo Scientific (Fairlawn, NJ). Cell Titer-Blue® Cell Viability Assay kit is obtained from Promega Inc. (Madison, WI). All other chemicals are ordered from VWR (Radnor, PA).

Methods

Nanoassemblr (micromixer) design and setup

Liposome formulations using the micromixer were prepared on a benchtop NanoAssemblr™ instrument in Dr. Gaurav Sahay's lab, College of Pharmacy, OSU (Precision NanoSystems Inc. BC, Canada). A disposable PDMS cartridge was used for the manufacturing of liposomes in the NanoAssemblr™. The cartridge is connected to two inlet streams. The two inlet streams are comprised of lipids dissolved in ethanol and aqueous magnesium sulfate pH 4 buffer. The flow rate ratio (FR) is the rate at which both streams pass through the channels in the herringbone structure, and the mixing ratio (MR) is the ratio at which both the aqueous and organic streams are mixed (v/v) as they pass through the channels. Both inlet streams are controlled by syringe pumps. A heating block was used to keep the solvents above the lipid transition temperature which is 60 °C for the spm/chol mixture.

Preparation and optimization of spm/chol vesicles using microfluidics

Preparation of empty liposomes

The organic phase consisted of spm and chol dissolved in 3 mL ethanol to attain four different molar lipid ratios (spm:chol), 100, 75:25, 55:45 and 25:75, to achieve a final volume of 5 mL with a

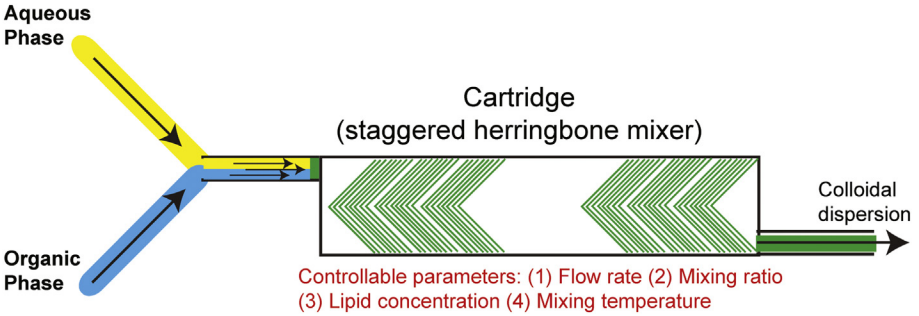


Figure 1. Schematics for preparation of spm/chol liposomes using microfluidics.

lipid concentration of 10 mM. Slight heat was required to dissolve the lipids, but once in solution, they were stable. The aqueous phase consisted of 300 mM $MgSO_4$ (pH 4). Based on the transition temperature of the lipid mixture, the heating block was set at 60 °C. At the end of each run, lipid vesicles were collected in the chamber outlet. The vesicles were then purified by passing them through a Sephadex PD 10 column pre-equilibrated with 300 mM sucrose, 3 mM EDTA, and 20 mM HEPES (SHE) buffer at pH 7.5. The final volume collected was 3.5 mL which was q.s. to 4 mL with SHE buffer. The empty liposomes were stored at 4 °C till further drug loading was performed. The final lipid concentration was 10 mM for all the lipid ratios.

Microfluidics has three main controllable parameters: mixing ratio, flow rate and concentration of lipid, along with mixing temperature dependent on the lipid mixture, the variation of which results in vesicles of different size and dispersity[22,23].[24] Mixing ratio (MR) represents the ratio of aqueous to organic solution which determines the final lipid concentration in the suspension, along with final size and polydispersity.[22–24] The flow rate (FR) of the aqueous and organic solutions controls the flow of the mixture through the series of staggered herringbone micromixers that allow for introduction of a chaotic flow profile.[22] The alterations to the FR of aqueous and organic solutions result in variations in polarity throughout the chamber, which dictates the final size and PDI of the liposomes.[25] The concentration of lipids and their ratios influence the encapsulation efficiency, stability and size of the final formulation.[22] (See Fig. 1)

The preparation of spm/chol vesicles was studied as function of four different parameters shown in Figure 2: a) effect of three different aqueous/organic MRs: 5:1, 7:1, 9:1 evaluated at five different FRs: 3, 6, 8, 10 and 12 mL/min at a fixed lipid concentration of 10 mM, b) effect of five different FRs: 3, 6, 8, 10 and 12 mL/min studied at a lipid concentration of 10 mM at the optimized MR, c) effect of four different lipid ratios studied at optimized FR and MR, and d) effect of five different total lipid concentrations: 7, 10, 15, 20 and 25 mM studied at the optimized MR, FR and lipid ratio (mol:mol). For all studies, the optimal condition was decided based on the value of mean particle size (Z-ave size) and polydispersity index (PDI) of the liposome. One-way ANOVA with Dunnett's Multiple Comparison post-test at a *P*-value of 0.05 using Graph Pad prism version 6.05 for Windows was performed for all conditions.

For all studies, the lipid solution was passed through a 0.2 μm filter and analyzed for size and PDI using dynamic light scattering (DLS), except for lipid ratios where cryo-TEM was also performed, as it is most accurate method to visualize lamellarity. The data are presented as Z_{ave} ± SD and PDI ± SD for four replicates.

In vitro cell viability assay

To assess the CPD100Li-M *in vitro* efficacy, the cell viability of CPD100Li-M was studied as a function of oxygen levels and compared to CPD100Li-E published previously.[20] ES2 cells were seeded at a cell density of 3000 cells/well using RPMI culture medium supplemented with 10% FBS and 1% penicillin/streptomycin in a 96-well culture plate. The growth condition for ES2 cells was 37 °C in a humidified atmosphere of 5% CO2 (v/v) in air. The cells were allowed to attach for 3 h. Post attachment, the cells were treated with 10 mM phosphate buffer (control), empty liposomes at 12.5 mM (vehicle control) and CPD100Li (0.02-100 μM) for a total of 72 h. The treatment was studied at two levels of oxygen concentrations: normoxic 20% and hypoxic 1.5% (v/v). After 18 h pre-incubation at either oxygen level, the plates were incubated for the remaining 54 h under 20% O2 and the cell viability was assessed using the CellTiter Blue® assay per manufacturer's instructions. Briefly, 20 μL of the reagent was added to each well, and the cells were incubated for 2 h at 37 °C. The fluorescence intensity was measured at $560_{Ex}/590_{Em}$.

Half-maximal inhibitory concentration (IC_{50}) was determined with non-linear regression analysis using GraphPad Prism version 6.05 for Windows, GraphPad Software, La Jolla, CA, USA. All experiments were performed in quadruplicate, and data are presented as mean IC_{50} ± SD. The IC_{50} values for all groups are compared to CPD100Li-E by one-way ANOVA with Tukey's Multiple Comparison post-test at a *P* value of 0.05 using Graph Pad prism version 6.05 for Windows.

Pharmacokinetics study

To evaluate the CPD100Li-M effect on the *in vivo* circulation time of CPD100, a pharmacokinetics study was performed in Swiss Webster female mice. The mice were randomly divided and dosed with CPD100Li-M at 30 mg/kg *via* IV tail injection. Terminal blood samples (~0.7 mL) were collected *via* cardiac puncture at 0, 0.25, 1, 2, 4, 8 and 24 h post dosing in chilled NaF/EDTA blood tubes, mixed *via* inversion and maintained on wet

Optimization of spm/chol microfluidics experimental design

	a. Optimization of MR			b. Optimization of FR	
	MR1	MR2	MR3	mL/min	Lipid conc. (mM)
	(aqueous: organic v/v)				
1	5:1	7:1	9:1	3	10
2	5:1	7:1	9:1	6	10
3	5:1	7:1	9:1	8	10
4	5:1	7:1	9:1	10	10
5	5:1	7:1	9:1	12	10

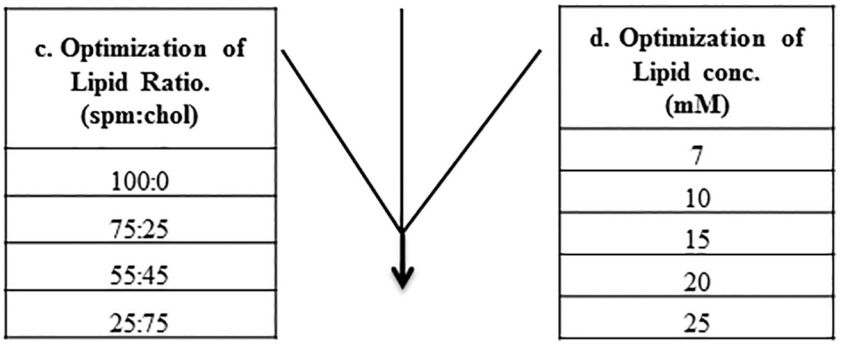


Figure 2. Experimental design for optimization of microfluidics conditions for spm/chol.

ice. For ensuring the stability of the sample, anticoagulated whole blood aliquot (0.25 mL) was added to a chilled cryovial containing 1 mL of cold 8.5% phosphoric acid and stored at −80 ± 5 °C until shipped for analysis. All bioanalytical analysis was performed by MicroConstants Inc. (San Diego, CA). The pharmacokinetics of CPD100Li-M in plasma was analyzed by non-compartmental analysis using Phoenix 64 (Certara, Princeton, NJ). The area under the concentration *versus* time curve from 0 to ∞ (AUC0-∞), plasma volume of distribution (Vd), clearance (CL) and half-life (t1/2) were calculated and compared to CPD100Li-E.

In vivo acute toxicity studies

All animal work was performed in compliance with NCI guidelines, Oregon State University and Oregon Health Science University IACUC Policy for End-Stage Illness and Pre-emptive Euthanasia, based on Humane Endpoints Guidelines. Swiss Webster mice were used to evaluate empty liposomes prepared using microfluidics and CPD100Li-M mediated acute toxicity. Previous work[20] with CPD100 and CPD100Li-E demonstrated a maximum tolerated dose (MTD) of 40 mg/kg for both formulations in Swiss Webster mice. Because we are comparing the toxicity of an optimized formulation, we injected the mice at the previously established MTD. Mice were injected with empty liposomes or CPD100Li-M *via* multiple tail vein injections (every three days) for 5 injections (n = 10; 5 animals/ group). The empty liposomes were dosed at a lipid dose of 50 mg/kg. The animals were monitored for weight loss and other signs of behavioral and physical toxicity for a period of 14 days. Data are presented as mean percent (%) normalized body weight ± SD.

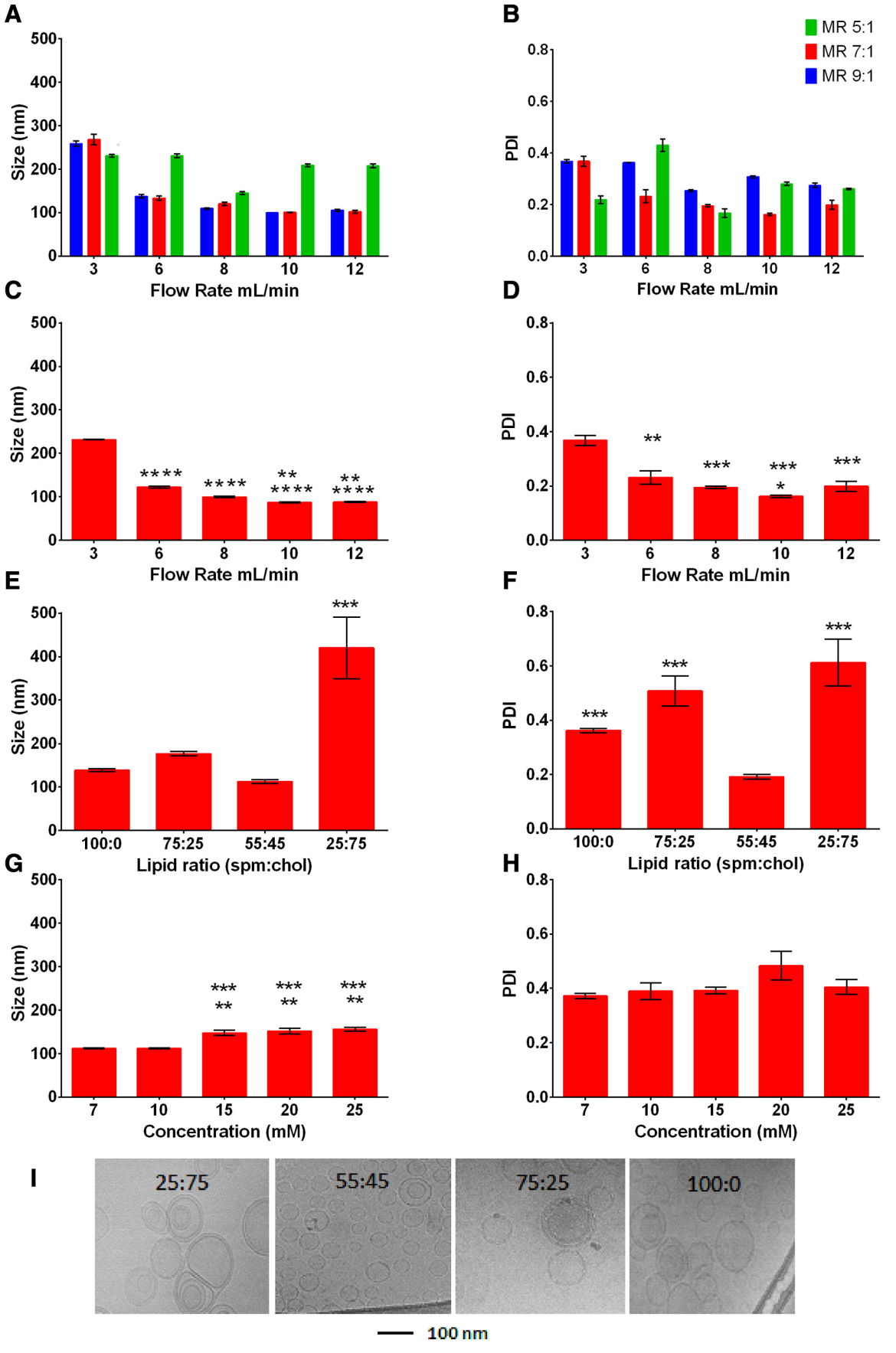
The detailed methods for preparation of CPD100Li-E and plasma preparation are described in the supplementary materials.

Results

Preparation and optimization of spm/chol vesicles using microfluidics

Preparation of empty liposomes

To determine the best MR and FR for microfluidic production of spm:chol (10 mM lipid concentration), we evaluated the size of the liposomes produced by varying the aqueous/organic MR from 5:1, 7:1 and 9:1 and FR from 3, 6, 8, 10 and 12 mL/min (Figure 3, *A* and *B*). The liposomes obtained at a MR 5:1 have a size of 230 nm and PDI of 0.2 at an FR of 3 mL/min. There was no significant change in size at higher FR at the 5:1 MR except at an FR of 8 mL/min where a lower size of 140 nm and a PDI 0.15 is achieved. However, a clear trend is seen for liposome size at higher MR of 7:1 and 9:1. At flow rates of 10 and 12 mL/min, smaller vesicles around 100 nm are achieved for both MR of 7:1 and 9:1. The PDI values are still high in the range of 0.25-0.35 for MR 9:1. The ideal vesicles obtained are 100 nm with a 0.16 PDI at MR 7:1 and FR 10 mL/min (Figure 3, *A*, *B*).


A
Size (nm)
Flow Rate mL/min
B
MR 5:1
MR 7:1
MR 9:1
PDI
Flow Rate mL/min
C
Size (nm)
Flow Rate mL/min
D
PDI
Flow Rate mL/min
E
Size (nm)
Lipid ratio (spm:chol)
100:0
75:25
55:45
25:75
F
PDI
Lipid ratio (spm:chol)
G
Size (nm)
Concentration (mM)
H
PDI
Concentration (mM)
I
25:75
55:45
75:25
100:0
100 nm

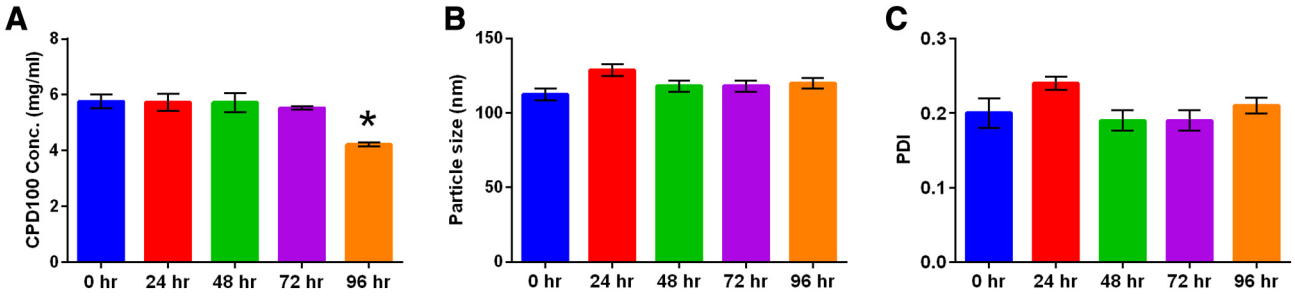


Figure 4. Solution stability of CPD100-loaded spm/chol liposomes as a function of time, **(A)** concentration,* indicates statistical significance as compared to concentration of CPD100 at 0 hr., **(B)** particle size (nm), and **(C)** PDI. Data are presented as mean values ± SD (n = 4).

At the optimized MR of 7:1 and lipid concentration of 10 mM, liposomes were formed at different FR of 3, 6, 8, 10 and 12 mL/min. When the FR was increased while maintaining a constant ratio between the organic and aqueous stream, the size was reduced from 230, 125, 100, 90 and 88 nm at an FR of 3, 6, 8, 10, 12 mL/min, respectively (Figure 3, *C*). Clearly, as the FR increased, the size decreased. The trend is also similar for PDI, with the highest equal to 0.38 at 3 mL/min, decreasing to 0.17 at 10 mL/min, and then increasing back to 0.21 at 12 mL/min (Figure 3, *D*). This confirms our previous conclusion that the FR of 10 mL/min at MR of 7:1 yielded the smallest particle size and PDI.

The optimized MR and FR parameters are used to examine the optimum lipid MR for generation of spm/chol liposomes. The size of the vesicles obtained for all the lipid ratios was below 200 nm except 25:75 which resulted in vesicles of 425 nm (Figure 3, *E*). The PDI at all lipid ratios were >0.2 except 55:45 (Figure 3, *F*). The size and PDI were also confirmed with cryo images which aligned with the above observation. The liposomes formed with spm:chol (55:45) show LUVs with uniform population distribution; on the other hand, other liposomes with different spm;chol ratios show MLVs with heterogeneous population distribution (Figure 3, *I* and supplementary results). Therefore the liposome with spm;chol 55:45 ratio is selected for further optimization and development. Liposomes with the optimized lipid ratio, MR and FR are used to optimize the lipid concentration. With lipid concentrations of 7 mM and 10 mM, smaller vesicles around 100 nm are produced. Vesicles around 150 nm are produced at higher concentrations of 15, 20 and 25 mM. Size of liposomes is independent in the range of 15-25 mM. The PDI is around 0.4 throughout the range of lipid concentrations studied (Figure 3, *G*, *H*).

Characterization of liposomes

CPD100-loaded spm/chol vesicles: stability assessment

CPD100 is loaded into spm/chol at a concentration of 5.8 mg/mL (Figure 4, *A*). As seen in Figure 4, *B-C*, CPD100Li-M has a size around 120 nm and PDI of 0.2 for a period of 96 h. However, at 96 h, the CPD100 concentration dropped to 4.3 mg/mL. CPD100 loaded into other liposomes at the four different spm: chol lipid ratios resulted in the following loaded concentrations: 100:0 (2.0 mg/mL), 75:25 (1.77 mg/mL), 55:45 (5.8 mg/mL) and 25:75 (0.8 mg/mL).

To achieve long term stability, CPD100Li-M is freeze dried following a previously established protocol.[20] As seen in Table 1, size measurements using three different techniques show no difference between the reconstituted freeze-dried (FD) formulation and CPD100Li-M in solution. The DLS shows a size of about 112 nm ± 4.09 nm with a PDI of 0.2. The PDI value signifies that the solution is monodisperse in nature. DLS is the gold standard for measuring size of nano-formulations; however, it does not measure the % population as a function of size, which gives an indication of size associated toxicity. Thus, tunable resistive pulse sensing (TRPS) analysis is performed to assess individual particle population and concentrations of CPD100Li-M. CPD100Li-M and FD CPD100Li-M have a total concentration of 2.9×10^{11} and 4.20×10^{10} particles/mL, respectively (Figure 5, *A*, *B*). The CPD100Li-M solution has a maximum population of about 14% with mean diameters of 112 nm and the FD formulation has 13% at 108 nm (Figure 5, *A*, *B*). Both the solution and the FD formulations have less than 0.5% of the vesicle population > 200 nm. The cryo-images for both states of CPD100Li-M show that the majority of the liposomal population is comprised of LUVs, as seen in Figure 5, *A*, *D*.

Overall, the size and CPD100 loading in liposomes prepared with microfluidics produced small liposomes compared to those prepared with extrusion[20] (Table 2). The DLS size for extruded liposomes is around 155.4 ± 4.15 nm compared to 112.5 ± 4.09 nm for microfluidics, but no difference was seen in the cryo-TEM size analysis; however this method is only qualitative. The loading for CPD100Li-M is 5.8 mg/mL compared to 5.5 mg/mL for CPD100Li-E.[20] The CPD100Li-M FD resulted in a loading of 5.6 mg/mL identical to CPD100Li-M.

Figure 3. Liposomes produced using microfluidic method with different MR, FR, spm:chol ratios and total lipid concentrations. Effect of the FR and MR on liposome size **(A)** and PDI **(B)** at 10 mM total lipid. Effect of different FRs on liposome size **(C)** and PDI **(D)** at MR (7:1). Effect of different lipid ratios on liposome size **(E)** and PDI **(F)** at MR (7:1) and FR (10 mL/min). Effect of different lipid concentration on liposome size **(G)** and PDI **(H)** at lipid ratio 55:45, MR (7:1) and FR (10 mL/min). Cryo-TEM images for the liposomes with the four different lipid ratios at FR 10 mL/min and MR (7:1) (I). Data are presented as mean values ± SD (n = 4). For panel **C**, **** indicates statistical significance as compared to FR 3 and 6 mL/min and ** indicates statistical significance as compared to 10 mL/min; for panel **D**, ** and *** indicate statistical difference as compared to 3 mL/min and * indicates statistical difference compared to 6 mL/min. For panels **E** and **F**, *** indicates statistical significance as compared to 55:45 spm/chol lipid ratio and for panel **G**, *** indicates statistical significance as compared to FR 7 mM and ** indicates statistical significance as compared to 10 mM lipid concentration.

Table 1
Size assessment of CPD100Li-M, liposomal dispersion freshly prepared or reconstituted form freeze- died powder.

Sample	Zave ± SD (DLS)	PDI ± SD (DLS)	Zave (qNano) (nm)	Cryo (nm)
Fresh	112.5 ± 4.09	0.21 ± 0.02	122	56.02 ± 13.2
Freeze-Dried	114.7 ± 2.8	0.16 ± 0.01	130	52.50 ± 8.12

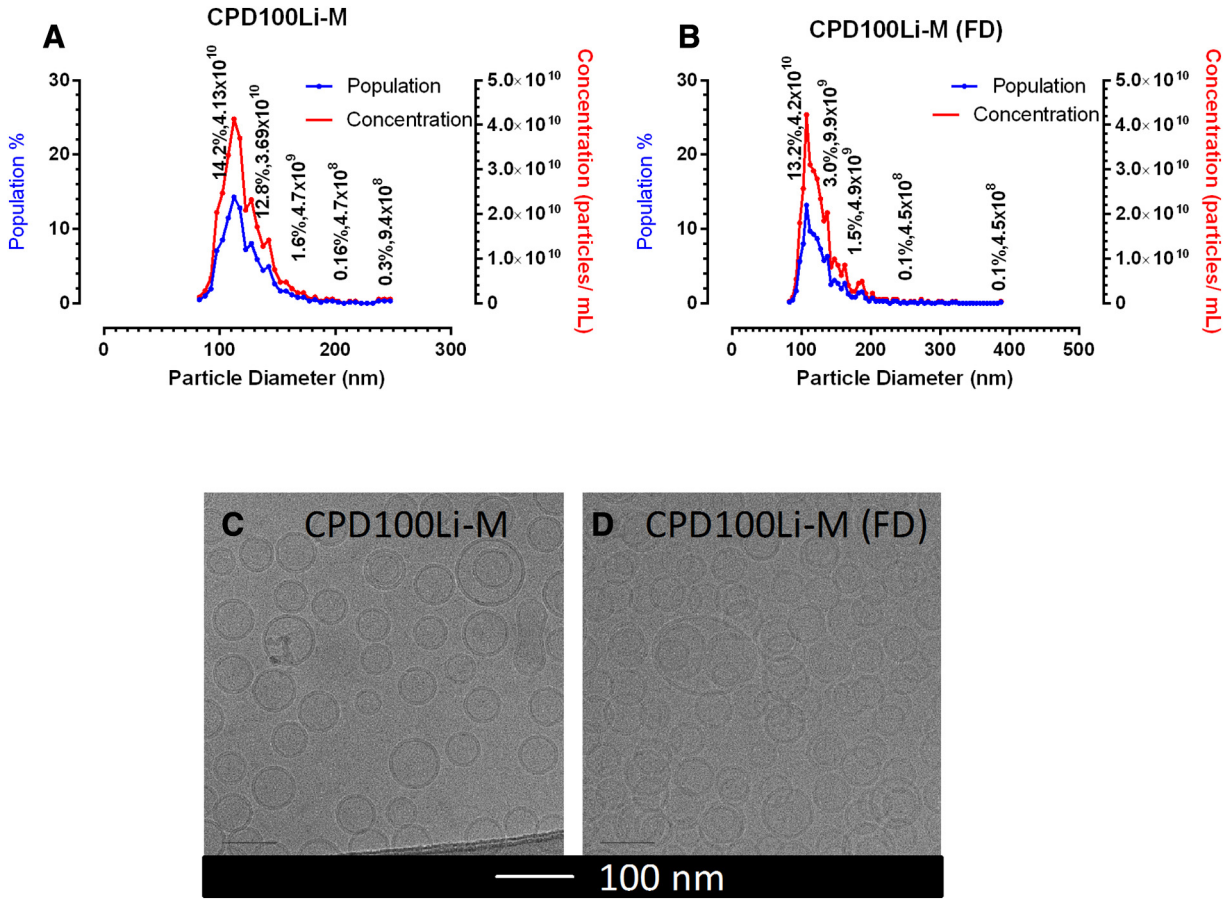


Figure 5. Mean particle size *vs* % population and particle concentration for **(A)** CPD100Li-M, and **(B)** CPD100Li-M (FD). Peaks in panels **A-B** are labeled with (Population %, particles mg/mL). Representative Cryo-TEM images for **(C)** CPD100Li, and **(D)** CPD100Li (FD).

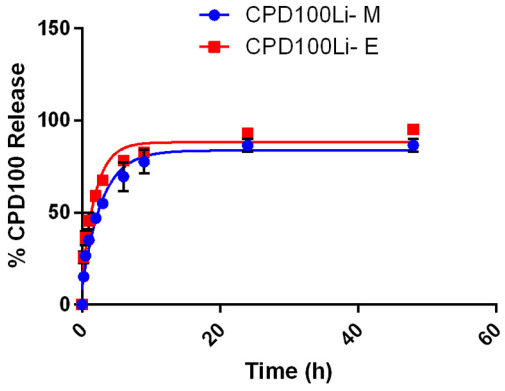
In vitro release kinetics

The release of CPD100 from liposomes prepared using both microfluidics and extrusion methods[20] is reported in Figure 6. The release of CPD100 from both liposomes is primarily in the first 6 h. The half-time for CPD100 release from liposomes prepared with microfluidics and extrusion is identical, with a goodness of fit value (r^2) of 0.97 and 0.95, respectively. The r^2 values indicate that the release of the prodrug from the liposomes followed a one-phase association with a calculated half-time of 1.99 h (CPD100Li-M) and 1.33 h (CPD100Li-E).[20]

A release study indicates the rate of release of the encapsulated molecule from the delivery system. The study can also be utilized as a quality control measure to ensure that there is no difference between the CPD100Li-M and CPD100Li-E.

Table 2
Size comparison of CPD100Li prepared using microfluidics and extrusion.

	Sample	Zave ± SD (DLS)	PDI ± SD (DLS)	Zave (qNano)(nm)	Cryo (nm)	CPD100 Conc. (mg/mL)
Microfluidics	**Solution**	112.5 ± 4.09	0.2 ± 0.02	122	56.02 ± 13.2	5.8 ± 0.25
	Freeze-dried	114.7 ± 2.75	0.16 ± 0.01	130	52.50 ± 8.12	5.6 ± 0.48
Extrusion[20]	**Solution**	155.4 ± 4.15	0.1 ± 0.02	132	64.26 ± 10.87	5.5 ± 0.37



	CPD100Li- M	CPD100Li- E
Half-time (h)	1.994	1.334

Figure 6. Release kinetics of CPD100 from CPD100Li-M and CPD100Li-E. Data are presented as mean ± SD of 4 replicates.

In vitro cell viability assay

To investigate if the anti-proliferative effect of CPD100 changed when formulated using a microfluidics platform, CPD100Li-M and CDPD100Li-M FD were evaluated in ovarian ES2 cells under 20% and 1.5% oxygen. This was then compared to CPD100Li-E, which had an IC_{50} of 32,100 ± 7159 nM at 20% and 3489 ± 1338 nM at 1.5% oxygen.[20] The IC_{50} values of CPD100Li-M and CPD100Li-M FD at 20% and 1.5% are depicted in Figure 7. The IC_{50} is 32,176 ± 740.2 nM for CPD100Li- M and 29,935 ± 7105 nM for CPD100Li-M FD. The values of CPD100Li-M and CPD100Li-M FD at 1.5% are 4242 ± 169.7 nM and 4641 ± 452.6 nM, respectively. Empty liposomes produced no reduction in cell viability of ES2 cells. There are no statistical differences between the IC_{50} values for CPD100Li-M, freeze dried CP100Li-M and CPD100Li-E at both 20% and 1.5% oxygen.

Previously published work has shown that the IC_{50} of CPD100 in ES2 ovarian cancer cells is 25,077 ± 9.17 nM and 4063 ± 1.5 nM at 20% and 1.5% oxygen levels, respectively.[20,26,27] Both the CPD100Li-M and CPD100Li-M FD have comparable IC_{50} values to CPD100, ensuring that the liposomal form does not change the anti-proliferative ability of CPD100. Also, previous work shows the IC_{50} value of CPD100Li-E in ES2 cells to be 32,100 ± 7159 nM (20% oxygen) and 3489 ± 772 nM (1.5% oxygen).[20] This indicates that there is no statistical difference in the anti-proliferative effect between the liposomes produced by extrusion or microfluidics.

Pharmacokinetics study

Liposome manufacturing method has an effect on liposomal *in vivo* behavior. Thus, to evaluate the *in vivo* characteristics of microfluidics-produced spm/chol liposomes, a pharmacokinetics study was performed. The plasma concentration-time curve of CPD100Li-M compared to the blood profile of CPD100Li-E following a 30 mg/kg single IV dose is shown in Figure 8 and Table 3. The CPD100Li-M pharmacokinetic profile showed a C_{max} of 49,550 ng/mL, AUC of 76,752 h*ng/mL and half-life of 5.9 h, whereas CPD100Li-E had a C_{max} of 54,300 ng/mL, AUC of 84,221 h*ng/mL and half-life of 5.5 h.[20,21] Pharmacokinetic data were not performed for the CPD100Li-M FD as no difference was seen in the size, PDI and CPD100 loading between the CPD100Li-M FD and the fresh CPD100Li-M.

In vivo acute toxicity studies

Maximum tolerated dose (MTD) is represented as a dose beyond which toxicity is seen in animals. Previous characterization

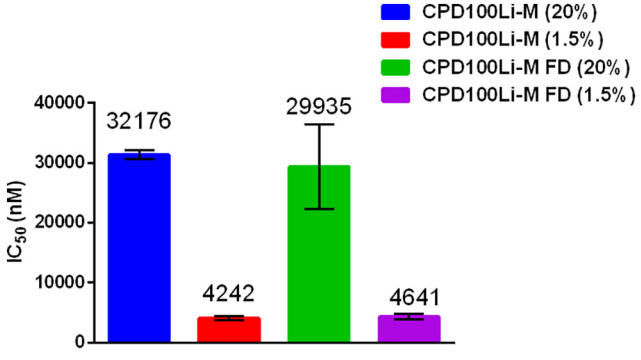


Figure 7. Mean IC_{50} values of CPD100Li-M and CPD100Li-M FD compared to CPD100Li-E[20] at 20% and 1.5% O_2 in ES2 cells ± SD (n = 4).

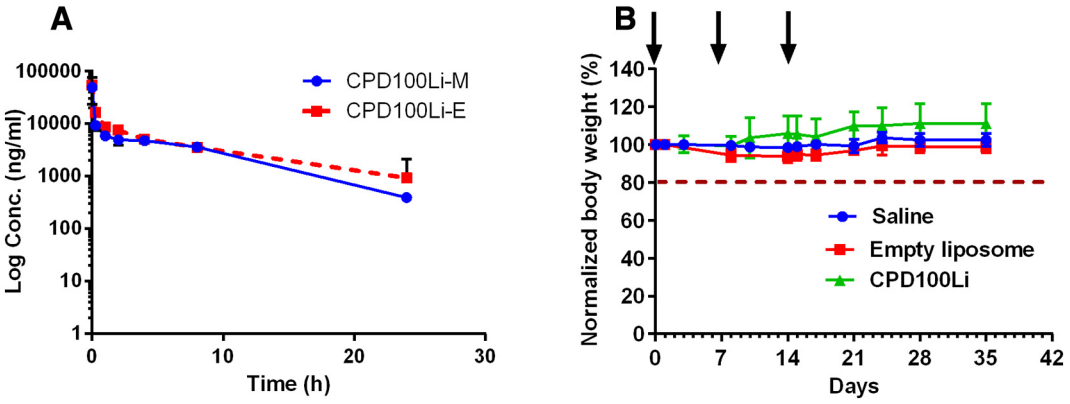


Figure 8. Plasma concentration as a function of time profile of CPD100Li-M and CPD100Li-E[20] in mouse plasma at a dose of 30 mg/kg **(A)**, and maximum tolerated dose evaluated as the normalized body weight as a function of days for control (saline), empty liposomes and CPD100Li dosed every week for 3 weeks **(B)**. Each data point is an average of 5 animals.

of the CPD100Li-E showed an MTD of 40 mg/kg which was identical to the MTD of CPD100 in both Swiss Webster and nude athymic mice.[20,21] To ensure that the empty microfluidics liposomes along with CPD100Li-M did not modify the MTD of CPD100Li, both lipid solutions were dosed in Swiss Webster mice once a week for 3 weeks. Empty liposomes were dosed at 50 mg/kg. The animals were observed for physical and behavioral changes. None of the treated mice during the study or 14 days after the study showed any signs of acute toxicity such as behavioral change, changes in eating habits/weight loss (> 20%) or death. The MTD in mice treated with CPD100Li-M was identical to those treated CPD100 and CPD100Li-E. The MTD study was not performed for CPD100Li-M FD because no difference was observed in size, PDI and concentration of CPD100 for the reconstituted CPD100Li-M and the fresh solution.

Discussion

Liposomes are an established drug delivery platform with several applications. The major limitation for the technology is lack of scale-up methods which can translate the processing parameters from the bench to clinic. Extrusion, in spite of numerous challenges with scale-up, is a standard method for preparation of liposomes. As a part of previously published work, we have performed extensive optimization of liposomes using the extrusion platform[20]; however, with challenges in scale-up optimization, we decided to evaluate and compare the liposomes prepared using extrusion to microfluidics. The results of this study provide an in depth comparison and conclusion that the *in vitro* and *in vivo* behavior of liposomes prepared using microfluidics is identical to extruded liposomes.

Table 3
Pharmacokinetic parameters of CPD100Li-M compared to CPD100Li-E.

PK Parameters		CPD100Li-M	CPD100Li-E[20]
$T_{0.5}$	h	5.87	5.5
AUC	h*ng/mL	76,752	84,221
Cmax	ng/mL	49,550	54,300

Microfluidics involves optimization of three main parameters: lipids mixing ratio, flow rate and concentration of lipid. It was observed that an increase in MR and higher FR resulted in smaller spm/chol liposomes, consistent with other liposomes generated using the microfluidics platform (Figure 3).[23] Overall, smaller vesicles are achieved with higher FR and MR.[23,24,28] Researchers have shown that the size and PDI are independent at lower FR of 2-4 mL/min for a number of lipids, but this is not valid for spm. The mixing ratio between aqueous and organic phases is a key parameter controlling the liposome size. Higher aqueous volumes favor formation of smaller lipid vesicles as the lipids get diluted in the aqueous solvent, resulting in faster mixing and reduction of organic solvent, leading to decreased particle fusion or Ostwald ripening.[29] In contrast, at low MRs, the organic solvent is injected into the system more slowly, allowing more time for both streams to interact, and therefore, producing larger liposomes.[29] The lipids also play a critical role in vesicle size and, in turn, stability of the liposomal formulation.[30] The effect of lipid concentration and ratio on the size and PDI aligns with published data where it has been observed that working with lipid concentrations greater than 3 mg/mL for most of the lipids result in vesicles around 100 nm.[28] Higher lipid concentration may lead to higher encapsulation at the cost of aggregation and associated toxicity.[9,10] The lipid ratio data highlight the importance of keeping cholesterol at least at 30 mole ratio and higher to result in a stable and rigid liposomal dispersion.[31] Moreover, the size and concentration which determine the clinical properties of the liposomes are only acceptable for lipid ratio 55:45, consistent with published work.[32–34] Size measurement, CPD100 loading and *in vitro* release (Figures 4-6, and Tables 1 and 2) not only dictate the efficacy of the liposomes, but are also an important quality control parameter to ensure reproducibility between batches. The overall goal is to develop liposomes with size around 100 nm and smaller PDI. Such platform can increase the CPD100 bioavailability; decrease the potential of hypersensitivity reaction C activation-related pseudoallergy which is associated with lipid colloid (size >300 nm)[35]; and potentially

take advantage of the enhanced permeability and retention (EPR) effect, which allows the drug-loaded liposomes to accumulate in tumor tissues.[36,37]

The cellular efficacy results indicate that the CPD100Li-M and CPD100Li-M FD achieved identical efficacy as the CPD100Li-E (Figure 7). Collectively, this shows that the preparation of liposomes using two different methods and freeze drying does not change the nature of cellular uptake and cellular efficacy of CPD100-loaded liposomes. The pharmacokinetic data, along with the MTD dosing study (Figure 8), clearly demonstrate that there is no difference between the *in vivo* characteristics of the CPD100Li manufactured using the microfluidics or extrusion method. In conclusion, the optimization data show the effect of various microfluidics parameters on the size and PDI of spm vesicles. The optimized CPD100 loaded microfluidics vesicles showed identical physical characterization in terms of size, PDI, and drug loading, along with identical IC_{50}, pharmacokinetic parameters and toxicity in animals, when compared to extruded liposomes. The work clearly highlights that both microfluidics and extrusion produce liposomes with identical *in vitro* and *in vivo* properties.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.nano.2019.02.019.

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