Comparing continuous micromixing and extrusion downsizing for PEGylated nanoliposomes remotely loaded with doxorubicin or the steroid pro-drug methylprednisolone hemisuccinate

Keren Turjeman, Alexander-Nicholas Egler-Kemmerer, Dima Shamrakov, Christine Paulus, Raphael Thiermann, Yechezkel Barenholz, Regina Bleul

PII: S0168-3659(25)00327-X

DOI: https://doi.org/10.1016/j.jconrel.2025.113707

Reference: COREL 113707

To appear in: *Journal of Controlled Release*

Received date: 7 October 2024

Revised date: 17 March 2025

Accepted date: 5 April 2025

Please cite this article as: K. Turjeman, A.-N. Egler-Kemmerer, D. Shamrakov, et al., Comparing continuous micromixing and extrusion downsizing for PEGylated nanoliposomes remotely loaded with doxorubicin or the steroid pro-drug methylprednisolone hemisuccinate, *Journal of Controlled Release* (2024), https://doi.org/10.1016/j.jconrel.2025.113707

Comparing continuous micromixing and extrusion downsizing for PEGylated nanoliposomes remotely loaded with doxorubicin or the steroid pro-drug methylprednisolone hemisuccinate.

Keren Turjeman[1#], Alexander-Nicholas Egler-Kemmerer[2#], Dima Shamrakov[3], Christine Paulus[2], Raphael Thiermann[2], Yechezkel Barenholz[1,*] and Regina Bleul[2,*]

[1] Laboratory of Membrane and Liposome Research, Department of Biochemistry, Institute for Medical Research Israel-Canada, Hebrew University-Hadassah Medical School, Jerusalem 9112102, Israel; kerenbarhum@gmail.com (K.T.); chezyb1@gmail.com (Y. B.)
[2] Fraunhofer Institute for Microtechnology and Microsystems IMM, Nanomaterials for Cancer Therapies, Business Division Chemistry, Carl-Zeiss-Strasse 18-20, 55129 Mainz, Germany; regina.bleul@imm.fraunhofer.de (R. B.)
[3] Ayana Pharma Ltd., Hadassah Ein Kerem Campus, JBP, POB 12133, Jerusalem 9112002, Israel; dima@ayanapharma.com (D.S.)

equal first authors; * equal senior authors, Correspondence: regina.bleul@imm.fraunhofer.de

Abstract:

Since the FDA approval of the first nanodrug Doxil® in 1995, twenty subsequent liposome-and lipid nanoparticle (LNP) based drugs (of which 10 are nanodrugs), were approved by the FDA. The application of such drug-products was considerably boosted by the mRNA-LNP based vaccines used to stop the COVID-19 pandemic. Research on lipid-based vesicles and nanoparticles for drug delivery dates to the 1970s and has culminated in both continuous flow and extrusion-based fabrication processes for current state-of-the-art GMP industrial production of nanoliposomes and lipid nanoparticles. In this study, we compare these two approaches for the preparation of two PEGylated nanoliposome-based drug-products, keeping all other production steps leading to the final drug-product identical. One of these products, generic Doxil®, is remotely and actively loaded with the anthracycline doxorubicin (an amphipathic weak base) driven by a transmembrane ammonium gradient, while the other is methylprednisolone hemisuccinate (an amphipathic weak acid) remotely and actively loaded via a transmembrane acetate gradient. We demonstrate that a microfluidics-based micromixer approach yields equivalent or even better drug-products, especially since the downsizing by microfluidic is not performed above the temperature range of lipid phase transition. The main difference in the physico-chemical features is that size distribution of the microfluidics prepared pegylated nano liposomes was significantly narrower and morphological analysis by cryo-TEM confirmed higher homogeneity. An additional advantage of the microfluidic approach is that it is a continuous production. Therefore, it enables the direct production of large volumes of high-quality nano-liposomal based drug- products.

Keywords:

nanomedicine; liposomal doxorubicin; drug loading; Doxil®; microfluidics; micromixer

1. Introduction

Drug delivery systems based on liposomes have been developed to improve the therapeutic index of novel or commonly used drugs by modifying drug absorption, reducing its metabolism, prolonging circulation time, and reducing toxicity [1]. The first and to date most widely used injectable drug delivery systems in clinical use are liposomes, which possess valuable therapeutic properties due to their biocompatibility, non-immunogenicity, biodegradability, broad spectrum of lipid compositions and versatile methods of production [2]. With 17 clinically approved liposome-based drug formulations (10 of which are nanodrugs), they are the leading injectable drug delivery system, mainly used in cancer treatment [3]–[9]. Recently new types of lipid-based nucleic acid (siRNA and mRNA) delivery systems, referred as lipid nanoparticles (abbreviated as LNPs), have been approved by FDA/EMA and introduced to the clinics – for silencing by siRNA (Onpattro) and for vaccination against COVID-19 using mRNA in the vaccine formulations Comirnaty® and Spikevax® [10]–[13]. Even though both nanoliposomes and LNPs are nanoscale and consist of polar lipids, they differ from each other. Liposomes are based on an envelope of a lipid bilayer that encapsulates the aqueous phase within the liposomes, in which low and high molecular weight water-soluble or amphiphatic drug substances are stably retain [14]. Therefore, liposomes can be successfully used for remote active loading with amphiphatic weak acids and bases, as shown previously [14] and in this manuscript. LNPs have a fundamentally different structure compared to liposomes since they are surrounded by a lipid shell, which can be either a bilayer or a monolayer. The latter is possible due to the lipidic core of the LNP. In the inner volume of the LNP, there is only a small aqueous phase within the particle, and most of the intra-LNP volume is occupied by the lipid core in which nucleic acids such as siRNA and mRNA are located. In addition, LNPs are leaky and cannot retain low molecular weight drug substances such as doxorubicin or methylprednisolone hemisuccinate and cannot generate neither pH nor ion gradients. Namely LNPs and liposomes are dealing with different aspects of drug delivery, and this constitutes the novelty of our MS; particularly in combination with the direct scalable continuous manufacturing of the nanoliposomes. The production of these three LNP drug-products does not involve an extrusion step and are based on a continuous manufacturing process.

The large-scale commercial production of the two mRNA-LNP anti COVID-19 vaccines in contrast to classical liposomal drugs involves the use of a similar T-mixing approach instead of extrusion. Analysis of Comirnaty size measurements by DLS and by cryo-TEM reveals rather broad size distribution having about 50 to 200 nm diameter [15]. Such broad size distribution is accepted for vaccines but not for drug-products administered systemically. It is not clear if the Onpattros commercial production is based on T-mixing or microfluidics.

Recent database queries show the increasing interest in the use of microfluidics as a key step in the production of nanoliposomes and LNPs [16]. However, most current scaled-up microfluidic approaches address upscaling through parallelization while other concepts such as internal upscaling are rarely or not discussed at all.

So far, most of the liposomal nanopharmaceuticals in clinical use are produced via extrusion-based manufacturing processes. To date, to our best knowledge there is no FDA or EMA approved liposomal drug-product that is manufactured using a microfluidic process on an industrial scale. This deficiency is the motivation behind our current study. The way used to determine the utility of large-scale microfluidics is to compare side by side the currently used extrusion-based production process of relevant nanodrugs as generic Doxil® with the microfluidic production approach to produce identical drug products. Particularly, it takes into consideration the controllability of product parameters, scalability to large-scale volumes, and the continuous control of the presented process. Those could be of high value for potential clinical applications of emerging (liposomal) nanotherapeutics.

For most systemic applications, liposomes smaller than 200 nm and preferably ≤100 nm are of particular interest, as they generally have advantages in terms of *in vivo* behavior (long circulation time, biodistribution and meet the requirement to benefit from EPR effect [14],[17]) and allow sterile filtration in contrast to larger liposomes [18],[19]. Even though the harmful side effects of chemotherapeutic treatment have been significantly reduced using Doxil® and its generic versions, doxorubicin is still frequently used as a free drug in clinics as first-line therapy and part of the justification not to

use Doxil® is the high cost of the liposomal drug-product [20]–[23]. Although, for generic manufacturing the establishment of new production processes are hindered, due to the high regulatory requirements and cost of proving *in vitro* bioequivalence as well as equivalent pharmacokinetics in human clinical trials. Thus, novel processes are less attractive for generic manufacturing of liposomal nanodrugs, but it may be attractive for the manufacturing of new nanodrugs since the process is better controlled, cheaper and faster as demonstrated in this study. Our microfluidic approach, that produces nanoliposomes "bottom-up" from an ethanolic solution of lipids, could fill the existing gap of an efficient route from R&D to large-scale production in nanomedicine. The presented micromixer-based platform technology provides a controlled and continuous production process of high-quality nanoliposomes that is bidirectionally scalable. Compared to liposome production by extrusion, the microfluidics-based approach is a continuous process that requires no further size reduction or homogenization steps to achieve the targeted liposome size of 100 nm or less in only a few minutes runtime [24]–[26]. The nanoliposome manufacturing process is based on the self-assembly of amphiphilic lipids, exploiting the exceptional fluidic control in a micro-structured mixing module [24],[27]. With this, we provide an attractive manufacturing alternative omitting the need to produce MLVs which requires downsizing by extrusion. Both the fabrication of MLVs and the multiple extrusion steps have to be performed at temperatures above the range of lipids phase transition temperatures to enable formation of intact nanoliposomes [14]. However, one-step production of nanoliposomes by microfluidics can be performed at temperatures below lipids phase transition as high ethanol content during the nanoformulation process is present. It is known that increasing the ethanol content reduces the transition temperature compared to lipids in aqueous solution [28]. In the case of both the manufacturing processes that followed the manufacturing of nanoliposomes were identical and included transmembrane ion gradient driven remote active loading. To evaluate if the microfluidic based approach can be applied to the production of a broad spectrum of remotely and actively loaded drug-substances we perform the comparison between the two approaches for the amphipathic weak base anticancer drug substance doxorubicin (DXR) and the amphipathic weak acid steroid prodrug methylprednisolone hemisuccinate (MPS) [29]–[32]. Scaling capabilities using internal adjustment of microstructural dimensions have a significant potential to facilitate the transition from laboratory to production scale.

2. Materials and Methods

2.1 Materials

Unless otherwise indicated, all chemicals and lipids were used as supplied by the vendor without further purification. Sucrose (EMPROVE® ESSENTIAL, Ph. Eur., BP, ChP, JP, NF), ammonium sulfate (EMSURE® ACS, ISO, Reag. Ph Eur.), calcium acetate hydrate (extra pure), Dowex™ 50 WX-4 cation exchange resin (Na^+ form, 200–400 mesh), Dowex 2X-800 anion exchanger and absolute ethanol (EMPROVE® EXPERT Ph Eur., BP, ChP, JP, USP) were obtained from Merck KGaA (Darmstadt, Germany). Doxorubicin hydrochloride (DXR) was purchased from Teva Pharmaceuticals (Israel). Methylprednisolone 21 hemisuccinate (MPS; Solu-Medrol®) was obtained from Pfizer. The lipid mixture of hydrogenated soybean phosphatidylcholine (HSPC), 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-*N*-[amino(polyethylene glycol)$_{2000}$] (DSPE-mPEG$_{2000}$) and cholesterol was provided partly commercially, but mostly free of charge by Lipoid GmbH (Ludwigshafen am Rhein, Germany). Triton® X-100 for liposome lysis was obtained from Carl Roth GmbH & Co. KG (Karlsruhe, Germany). The highly pure water used for the drug stock and salt solutions was dispensed from a Milli-Q Plus purification system (Merck Millipore) with a QPAK® 2 purification cartridge (Merck KGaA, Darmstadt, Germany). All solutions were filtered through sterile filters (Stericup Filter Unit Millipore Durapore 0.22 µm) from Merck KGaA before use in the micromixer to prevent contamination-induced clogging.

2.2 Methods

2.2.1 Liposomes Fabrication

Nanoliposomes were produced continuously by controlled self-assembly *via* microfluidic mixing using a Fraunhofer slit-interdigital micromixer or a split-and-recombine micromixer with inner dimensions from 45 µm to 600 µm. In preparation for the assembly reaction, two reaction fluids were prepared for each experiment. For the lipid solution (up to 200 $g \cdot L^{-1}$ mass concentration), the ready-to-use lipid mix consisting of hydrogenated soybean phosphatidylcholine (HSPC), *N*-(carbonyl-methoxypolyethylene glycol$_{2000}$)-1,2-distearoyl-*sn*-glycero-3-2-phosphoethanolamine (mPEG$_{2000}$-DSPE) and cholesterol in a weight ratio of 3:1:1 was dissolved in moderately pre-heated absolute ethanol until the solution was clear and sterile filtered immediately before use. As a directing solvent, a sterile filtered aqueous salt solution (200, 250 and 300 mM respectively) of either calcium acetate or ammonium sulfate was blended with the lipid solution under lamellar flow conditions and temperature control in the mixing unit. Reagent and rinse solutions were delivered either via HPLC pumps with downstream back-pressure valves or syringe pumps at total flow rates (TFR) ranging from 10 $mL \cdot min^{-1}$ to 100 $mL \cdot min^{-1}$, where individual flow rates were adjusted to achieve at least a 1:1 dilution in the first mixing step. Flow rate ratios (FRR) from 1:1 to 1:10 were tested. The final ethanol concentration was 20% (v/v).

Exchange of extraliposomal medium for the generation of a transmembrane salt gradient was performed by continuous tangential flow filtration (TFF) using a TFF ultrafiltration system consisting of a Quantum Pump (Watson-Marlow, Falmouth, United Kingdom) equipped with a sterile disposable ReNu SU technology cartridge and an UFP-500-C-4A Hollow Fiber Cartridge (650 cm^2, pore size 500000 NMWC) from Cytiva. The ethanol content of the dispersion was lowered by diafiltration against the salt solution corresponding to the product, followed using an osmotically equivalent and low-ion 10% sucrose solution to establish the transmembrane salt gradient. Diafiltration process was monitored by conductivity measurements as described in 2.2.2.4..

Liposome drug loading based on the transmembrane salt gradient was performed as described elsewhere [29],[33]–[35]. Briefly, diafiltrated PEGylated nanoliposomes exhibiting the desired ion-gradient (ammonium sulphate or calcium acetate) were incubated with the targeted drug substance (DXR or MPS) in the appropriate aqueous solution and heated to accelerate the API uptake. Non-encapsulated drug was removed by dialyzing the drug-loaded liposomes against 10 mM L-histidine buffered (pH 6.5) 10% sucrose solution at 4°C.

2.2.2. Liposomes Characterization

2.2.2.1 Liposome Size by Dynamic Light Scattering (DLS)

DLS measurements performed in Mainz (Germany) were done using a Zetasizer Ultra Red instrument (Malvern Panalytical, Malvern, United Kingdom) and in Israel with a Zetasizer nanoZS (Malvern Panalytica) equipped with a He-Ne laser (λ = 633 nm, 173° detection angle) at 25 °C with an equilibration time of 120 s. A separate study done in Jerusalem (Israel) for generic Doxil® demonstrated an excellent equivalency of size determination between the used measuring devices. Samples were diluted 1:50 (v/v) in sterile saline (0.9% NaCl) or sterile 10% sucrose solution and analyzed in polystyrene disposable cuvettes (Brand GmbH + Co KG, Wertheim, Germany). Cumulated particle size, intensity weighted size distribution histograms, Z-Average of hydrodynamic size, polydispersity index (PDI) and percentiles (D_i10, D_i50, D_i90) were calculated based on autocorrelation functions with automated position and attenuator adjustments at multiple scans using the ZS Xplorer software and reported accordingly. SPAN was manually calculated based on the formula

$$(D_i90 - D_i10) \cdot D_i50^{-1}$$

2.2.2.2 Lipid Concentration Using HPLC-ELSD

Lipid content was quantified using a high-performance liquid chromatography-evaporative light scattering detector (HPLC-ELSD) method [36], which provides rapid and simultaneous quantification of lipid concentrations in liposomal systems.

2.2.2.3 Loading Efficiency

The efficiency of doxorubicin remote loading was determined photometrically using a UV-Vis spectrophotometer (Genesys 10S UV-Vis, Thermo Scientific, MA, USA) based on the method of Amselem *et. al* [37] by comparing the specific absorbance of drug molecules at 500 nm before and after treatment with the Dowex-50 cation exchange resin. Measurement at 600 nm served to compensate for absorbance of empty liposomes.

Quantification of steroid prodrug was done using HPLC as previously described [38]. The concentrations of intraliposomal MPS were obtained after treatment with Dowex 2X-800 anion exchanger for binding free drug molecules. Briefly, a HPLC system (Hewlett-Packard Series II 1090) using an RP-c18 (Alltech) column and operating at a flow rate of 1 mL · min^{-1} was used. Equilibration was done with a mobile phase comprising 33% acetonitrile and 67% 0.075 M sodium acetate buffer, which was adjusted to pH 5.8. Detection of MPS and its hydrolysis products was achieved using a UV detector set at a wavelength of 245 nm.

2.2.2.4 Determination of salt concentration with conductivity measurement

Conductivity measurements were performed using a digital conductivity meter (type GMH 3430, GHM Mess Technik GmbH, Germany) equipped with a two-electrode special graphite conductivity cell with integrated temperature sensor. The determination of the salt concentration from the conductivity measurements was carried out as described elsewhere [39] at constant temperature (22°C) using a calibration curve of 0.016–100 mM salt in 10% sucrose (conductivity range 12.5 µS–15.5 S).

2.2.2.5 Cryogenic Transmission Electron Microscopy (Cryo-TEM)

Morphology of nanoparticles and drug loading performance verification were analyzed by Cryo-TEM (N~1000 particles) as described in detail previously [40]. Briefly: samples were prepared by loading a 3 µL drop of particle dispersion onto a glow-discharged lacey carbon-coated copper grid (200–400-mesh), blotted with filter paper and immediately vitrified in liquid ethane at –180°C. The imaging process was performed under liquid nitrogen cryo-conditions using a Gatan UltraScan® CCD camera. Analysis of the acquired images was performed using QuTEM AB (formerly Vironova BioAnalytics AB, Stockholm, Sweden) analyzer software, with particle identification carried out automatically and manually for correction purposes.

2.2.2.6 Determination of Ethanol Content using Head-Space Gas Chromatography (HSGC)

The ethanol content of liposome dispersions was determined by HSGC technique, using an Agilent 7890 gas chromatograph with an FID detector, coupled with an Agilent G1888 transfer line instrument and Restek Rtx®-BAC1 30-meter 0.53-mm 3-µ film thickness capillary column. Injection was performed from headspace volume at 40 °C sample temperature with an injector temperature of 200 °C and an oven temperature of 40 °C, Calibration was performed with 500 ppm and 1000 ppm ethanol in water.

2.2.2.7 Differential Scanning Calorimetry (DSC) Measurements

The thermotropic behavior and thermodynamic parameters of the PEGylated nanoliposomes with exhibiting transmembrane ion gradient prepared by microfluidics and by extrusion with the same lipid mix batch were compared via DSC measurements by using a MicroCal™ PEAQ-DSC (Malvern Panalytical, Malvern, United Kingdom). Detailed information about the DCS method and DSC thermograms analysis can be found elsewhere [41]. In short, samples with 20 mg/mL lipid concentration and 10% sucrose as reference were scanned over a temperature range from 15 °C to 90 °C with a heating rate of 1 °C · min^{-1} to ensure near-equilibrium conditions. Data analysis was performed using MicroCal™ PEAQ-DSC software. A spline baseline correction was applied, and the thermograms were fitted using a non-two-state model to extract relevant thermal parameters: T_m, the temperature

of maximum change in the heat capacity; $\Delta T_{1/2}$, the temperature range at half height of endotherm which is related to level of cooperativity and ΔH, the phase transition induced change of enthalpy.

3. Results

This study demonstrates the feasibility to continuously manufacture high-quality nanoliposomes designed for transmembrane ion gradient driven the remote active drug-loading using a micromixer-based approach to accelerate the production process for liposomal drug-products. As illustrated below, the current production process of remotely loaded nanoliposomes by conventional multi-step batch process could be improved by introducing a continuous micromixer technique to replace multilamellar vesicle (MLV) production and their downsizing by extrusion. This shows that the microfluidic approach has the potential to simplify and improve the efficiency of nanoliposome production compared to the traditional extrusion-based method (Figure 1).

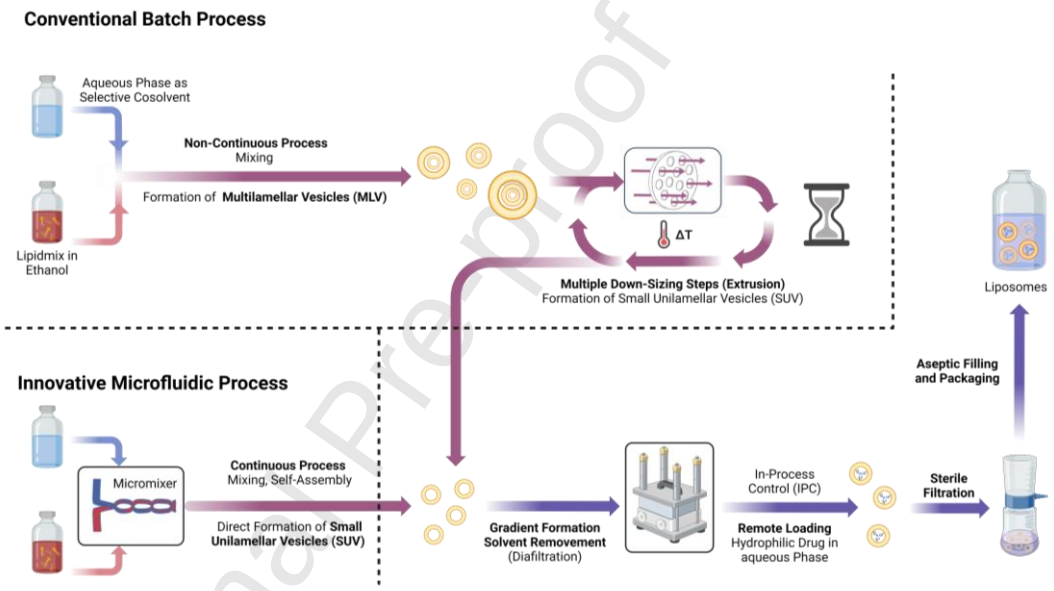


Figure 1. Schematic comparison of the conventional liposome production method using the batch process with MLV production followed by extrusion steps (top) for downsizing and the microfluidic approach presented here (bottom) using a micromixer, (instead of extruder) in which the desired nanoliposomes are produced by one step and without the need to work above the lipid phase transition temperature. Created in BioRender. Egler-Kemmerer, A. (2024) BioRender.com/r53z227.

3.1 Preparation and Characterization of Nanoliposomes

Nanoliposomes with either ammonium sulfate or calcium acetate transmembrane gradient were fabricated by the cosolvent method using a micromixer set-up and subsequent tangential flow filtration for ethanol removal and gradient formation. The desired target size (Z-Average) expressed as hydrodynamic diameter in the range of 80±10 nm and desired narrow size distribution with PDI below 0.1 and/or SPAN <0.75 were achieved *in situ* by one step during the self-assembly process. No subsequent downsizing or homogenization step was required.

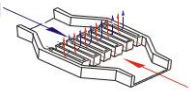
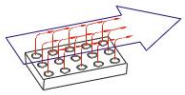
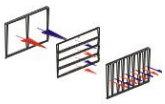
We have tested different micromixers with various flow profiles and dimensions (parameters summarized in Table 1). Depending on the total flow rate and mixing ratio, either a slit interdigital micromixer (SIMM, Table 1: A) with multilaminar flow profile or a caterpillar micromixer with split-and-recombine mixing profile was used. The small-scale set-up was equipped with syringe pumps for liquid supply whereas HPLC pumps were used for larger volumes.

The slit interdigital micromixer lab set-up was used for small flow rates of up to 5 mL · min^{-1} TFR. The used SIMM mixer consists of two comb-like microstructures (with 15 interdigitated microchannels with a width of 45 µm) for the two inlet streams, which are combined to a single stream and

focused through an outlet slit. This micromixer provides very rapid and efficient mixing and is therefore suitable especially for small flow rates. However, due to the small internal microstructure, there is an increased risk of channel blocking if uncontrolled precipitation occurs during the mixing process. To scale-up this particular mixing profile via multiple lamination, production processes can be transferred from slit interdigital microstructures to interdigital disk arrays (Table 1: B), enabling the realization of flow rates ranging from 12 to 30,000 L · h^{-1}.

Alternatively, the split-and-recombine micromixer was investigated in two different sizes. This micromixer also leads to a multi-lamination of two parallel inlet fluid streams entering a caterpillar-like microstructure (CAT, 300 µm or 600 µm inner diameter, Table 1: C), where each mixing element divides the incoming fluid stream into two lamellae and recombines them. With every mixing step, the number of lamellae increases by a factor of two. That means, for instance, that a micromixer with 12 mixing elements leads to 2^{12} lamellae resulting in multi-lamination and thus in a reduction of diffuse paths and accelerated mixing by maintaining a laminar flow. Compared to SIMM, the CAT micromixer enables a simple upscale possibility by increasing of inner dimensions. The mixing principal of CAT is a split-and-recombine mixing. Here, with every mixing step the number of fluid lamellae is doubled, thus thickness of lamellae is halved. As mixing occurs through diffusion the mixing time is dependent on the diffusion pathway which is identical with the thickness of the fluid lamellae. If the inner dimension of the CAT is increased, the required number of mixing steps needs to be increased as well. However, simulation work has shown that mixing is very efficient and already complete mixing occurs in the first third of the mixing chamber. Without increasing the mixing steps also for larger CAT versions, the mixing efficiency is sufficient to ensure a complete mixing withing the mixing chamber at higher flow rates. The mentioned simulation results can be found in reference [42]. Moreover, this micromixer is less sensitive to clogging.

Table 1. Comparison of the technical parameters of all tested micromixers for continuous liposome fabrication.

Micromixer	**A): Slit Interdigital Micromixer**	**B): Interdigital Disc Array**	**C): Caterpillar Micromixer**
Photograph			
Mixing profile			
Mixing principle	Multilamination	Multilamination	Split-and-Recombine
Nanomedical Applications	Small scale research (controlled self-assembly)	Lab to production scale (emulsion)	Lab to production scale (controlled self-assembly)
Throughput	≤1 L · h^{-1}	12–30.000 L · h^{-1}	0.5–250 L · h^{-1}
Channel dimension	45 µm	50–250 µm	150–2400 µm

Due to the scalability of the caterpillar mixer, the focus of our experiments was on its use and optimization of necessary reaction parameters. Size and size distribution were measured by Zetasizer Ultra Red. The polydispersity (<0.05) and SPAN (<0.75) were significantly improved compared to the experiments in the slit interdigital mixer with non-optimized reaction management. The SPAN of 0.94 achieved in the SIMM experiment is due to low D_i10 and the excessively high D_i90 value, which

is the result of an unoptimized flow rate ratio. Flow conditions in caterpillar micromixers were optimized in the ranges of total flow rate (10 to 100 mL · min^{-1}) and flow rate ratios (1:1 to 1:5) in the first mixing step, as the final ethanol content must be quickly reduced below 20% to achieve an initial stabilization of the formed liposomes. Thus, if a symmetrical mixing ratio is chosen, a direct subsequent dilution step must be included. Nanoliposomes with a salt gradient of ammonium sulfate or calcium acetate were successfully prepared at various scales (TFR of 10, 20 and 100 mL · min^{-1} respectively) with different micromixers. Table 2 summarizes the obtained results for the relevant parameters.

Table 2. Comparison of physicochemical characteristics of nanoliposomes with ammonium sulfate gradient obtained with different micromixers at various flow rate scales.

Micromixer	TFR	Size [nm]					PDI	SPAN
	[mL · min^{-1}]	Z-Average	Mean	D_i10	D_i50	D_i90		
SIMM	10	78.7	84.9	51.9	80.3	125.3	0.08	0.94
CAT300	20	79.6	82.8	60.1	81.0	109.4	0.01	0.68
CAT600	100	78.9	81.2	56.5	78.8	110.6	0.04	0.72

After self-assembly in the micromixer set-up, the liposome dispersions were further processed by tangential flow filtration (TFF) or dialysis for small scale to exchange the outer medium with sucrose to create the transmembrane gradient for subsequent remote drug loading. During TFF, possible size changes were monitored by online DLS, and final transmembrane ion gradient was verified by conductivity measurements that represent extraliposomal medium ion concentration. Medium exchange was stopped as conductivity indicated a value below 100 µS. Figure 2 shows a cryogenic transmission electron microscopy image of a representative sample of nanoliposomes exhibiting ammonium sulfate gradient prepared under optimal conditions before remote drug loading. The mean liposome size of 71.8 nm with SPAN of 0.28 (D_i10 = 63.2 nm, D_i50 = 69.2 nm, D_i90 = 82.8 nm) was measured using QuTEM AB VAS software. The fact that D_i50 is like the Z-Average and the exceptionally small SPAN indicates that the size distribution around D_i50 is narrow and homogeneous.

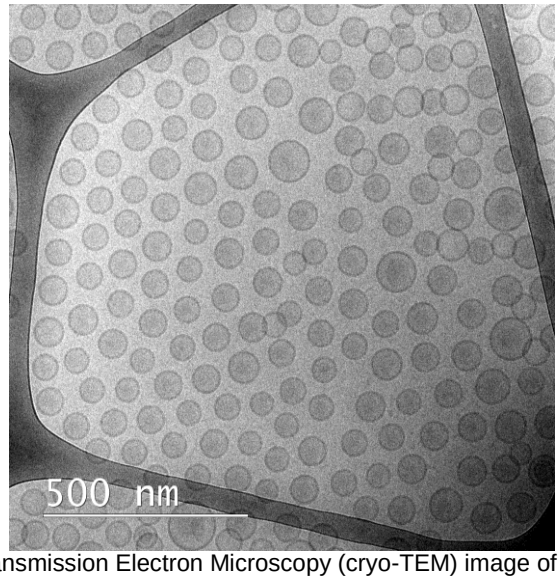


Figure 2. Cryogenic Transmission Electron Microscopy (cryo-TEM) image of continuously manufactured liposomes with CAT300 micromixer before DXR remote loading as representative example.

Depending on the saline solution used as a selective solvent, the final size of the liposomes differed slightly by a few nm. Furthermore, the TFF process with sucrose as well as storage at RT or 4°C also had a subtle influence on the size, which changed marginally during these measurements. The

size differences in the DLS measurement might also be affected by viscosity changes if the ethanol content was different. Moreover, the external medium (sucrose, calcium acetate or ammonium sulfate) also impacts the hydration state of the PEG lipid, which affects the hydrodynamic diameter determined in DLS measurements.

3.2 Drug Loading, Encapsulation Efficiency and Final Product Characteristics

Downstream processing was optimized for both ion gradients concerning conductivity, osmolality, and the final ethanol content after dialysis or diafiltration at room temperature. Desalination of liposome dispersion was performed by continuous TFF using a hollow fiber ultrafiltration system, a single-pass TFF module (Cadence®) or dialysis separation layer with 14 kDa cutoff (Membra-Cel™) for small scale workup (4 x 2 h exchange interval using 50-fold medium volume). For liposome stability increasement, the ethanol content of the dispersion was lowered by dilution before TFF processing. TFF was performed for two hours according to manufacturer's protocol, e.g. for operation of a module with 0.28 m² surface area with a starting volume of 20 L and recirculation and permeate flow rates of 8000 · mL min^{-1} and 116.7 mL · min^{-1} respectively. Finally, the extraliposomal medium is exchanged with an osmotically equivalent and ion-poor 10% sucrose solution to establish the transmembrane salt gradient. Hydrodynamic size as well as conductivity was monitored during the workup process: immediately after formulation, diafiltration and drug loading. One selected sample of each salt (acetate or sulfate) was further processed and exemplarily drug loaded.

3.2.1 Drug Loading with Methylprednisolone Hemisuccinate (MPS)

Nanoliposomes prepared using a micromixer (CAT300) and assembled in the presence of calcium acetate were characterized before and after TFF and active remote loading with MPS, respectively (**Table 3**). The average diameter of liposomes before and after diafiltration was 80 nm and 81 nm respectively with a relatively narrow size distribution (PDI ≤0.02, SPAN ≤0.7). Remote loading was performed as previously described [29]–[33],[42],[44] resulting in 92% pro-drug encapsulation (4.8 mg · mL^{-1} encapsulated MPS and 0.4 mg · mL^{-1} free, extraliposomal MPS). Liposome diameters did not change significantly (Z-Average = 81 nm) and the nanoliposomes remained spherical according to cryo-TEM. Zeta potential measurement of liposomal MPS indicated slight negative charge (–19 mV) related to the monoester phosphate of the DSPE-PEG [45]under low ionic strength conditions and neutrality (0.87 mV) under high ionic strength conditions.

At the final stage of diafiltration, the formulation was concentrated to 81.5 mg · mL^{-1} lipid. During remote loading, the target concentration was approximately 40 mg · mL^{-1}. The lipid composition, assessed with HPLC-ELSD, remained consistent with the initial lipid ratios. Mixing of lipids that have very different critical micelle concentrations (CMC) or critical aggregation concentrations (CAC), such as phosphatidylcholine and cholesterol, which have very low (in the range of nanomolar) CMC or CAC, with PEGylated lipids (e.g. DSPE-PEG), that have much higher values (1,000- to 10,000-fold) is very demanding of the mixing process. High dilution during the process and high ethanol concentrations might lead to a significant loss of the PEGylated lipid to the medium and the original molar ratio between the three lipidic components might change. However, this was not the case in the microfluidic based production as initial lipid ratios were fully recovered in the final drug-products (

Table 3).

Table 3. Physicochemical characterization of liposome samples with calcium acetate gradient directly after formulation, after diafiltration and after remote drug loading with MPS. Size, size distribution and Zeta potential were measured using a Zetasizer nanoZS.

	Parameter	Formulated	Diafiltrated	Remote-loaded
Size [nm]	Z-Av	80.1±0.13	80.8±1.20	80.5±1.40
	PDI	0.01	0.03	0.03
	D(i10)	59.4±0.80	59.9±1.00	59.8±1.10
	D(i50)	81.8±0.30	82.8±1.10	82.7±1.50
	D(i90)	114±3.00	115.7±1.70	115.3±2.80
	SPAN	0.67±0.04	0.69±0.03	0.67±0.03
Zeta Potential [mV]	ZP^1	-	-17.30^{*}	-19.40^{*}
	ZP^2	-	-1.03^{**}	0.87^{**}
Lipid Content [mg · mL^{-1}]	Total	61.5	81.5	43.4
	a) HSPC	36.3	47.6	25.4
	b) $mPEG_{2000}$-DSPE	12.6	17	9
	c) Cholesterol	12.6	16.9	9
	Weight ratio (a/b/c)	2.9/1/1	2.8/1/1	2.8/1/1

*Measured in a low conductivity medium (1.5 mM $NaNO_3$).

**Measured in a high conductivity medium (150 mM $NaNO_3$).

Stability testing conducted over one month confirmed the maintenance of 92% encapsulated drug. Cryo-TEM images (Figure 3) revealed the presence of a nano-precipitate of MPS calcium salt in the intraliposomal aqueous phase upon MPS loading, consistent with prior observations for liposomal-MPS produced using the ethanol injection technique and downsized *via* extrusion [46].

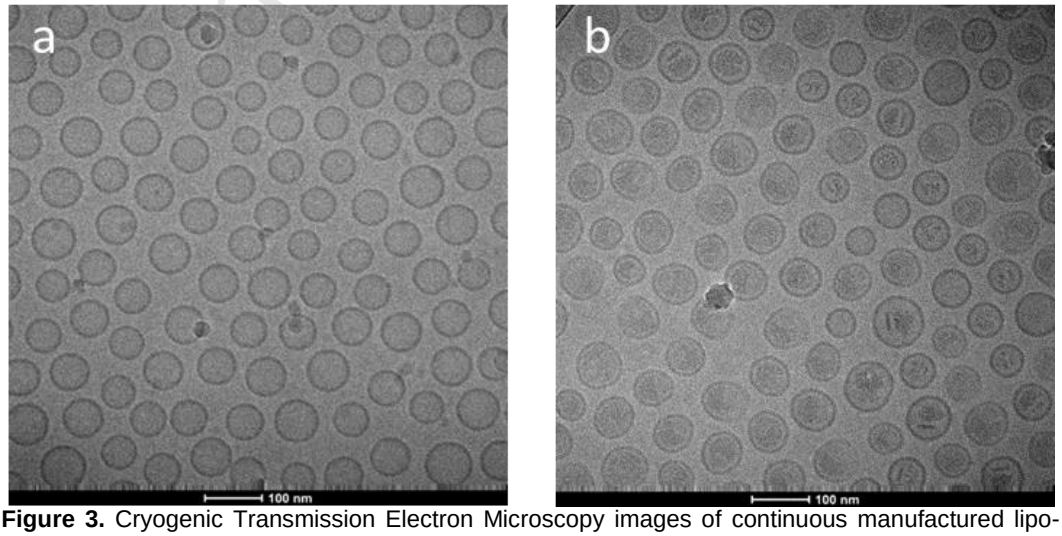


Figure 3. Cryogenic Transmission Electron Microscopy images of continuous manufactured liposomes before (a) and after (b) remote loading with MPS.

We conducted a comparative analysis of the release kinetics of MPS between the microfluidic produced PEGylated nanoliposomes and the extrusion-based PEGylated. The release kinetics, as depicted in Figure 4, demonstrated remarkable similarity, with a slightly slower release rate from the microfluidic-based liposomes (t½ of 90 h) compared to the conventional liposomes (t½ of 75 h).

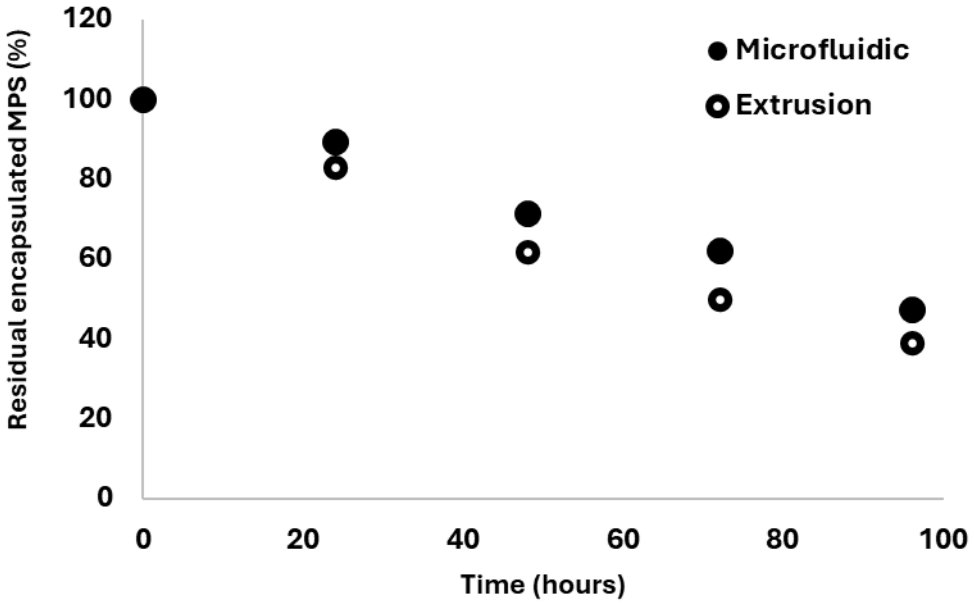


Figure 4. Comparison of release kinetics ("dissolution" assay) of MPS at 37 °C in human plasma from nanoliposomes produced using a microfluidic based process and conventional extrusion. Each point in the graph represents an individual measurement.

The process temperature of the liposome production plays a major role in their successful formation. Generally, liposomes are manufactured and down-sized above the transition temperature range of the specific lipid composition. However, in the case of the microfluidic production the lipids are dissolved in pure ethanol and are then mixed with the aqueous buffer system. The initially high ethanol content is reduced during mixing and subsequently completely removed after liposome formation. It is reported that the addition of solvent (e.g. ethanol) reduces the transition temperature of lipids significantly until non-detectability [28]. Therefore, the microfluidic produced liposomes were in fact handled below the transition temperature of the specific lipids and despite arranged similar like the liposomes from extrusion procedure. DSC measurements have been performed to confirm this statement experimentally.

The comparison of the thermotropic behavior studied by DSC between the PEGylated nanoliposomes prepared by extrusion and by microfluidics is described in Table 4 and Figure 5. Both the extrusion and microfluidic methods result in DSC thermograms characterized by a single, relatively broad phase transition that exhibits a low enthalpy endotherm. However, PEGylated liposomes produced via extrusion exhibited a T_m (the temperature of the maximum change in the heat capacity at the range of the phase transition) of 52.1 °C, an enthalpy change (ΔH) of 2.00 kcal · mol^{-1}, and a transition half-width ($\Delta T_{1/2}$) of 14.78 °C. In contrast, PEGylated liposomes

fabricated by the microfluidics approach showed a slightly higher T_m of 54.4 °C, a lower ΔH of 1.63 kcal · mol^{-1}, and a significantly narrower $\Delta T_{1/2}$ of 10.15 °C

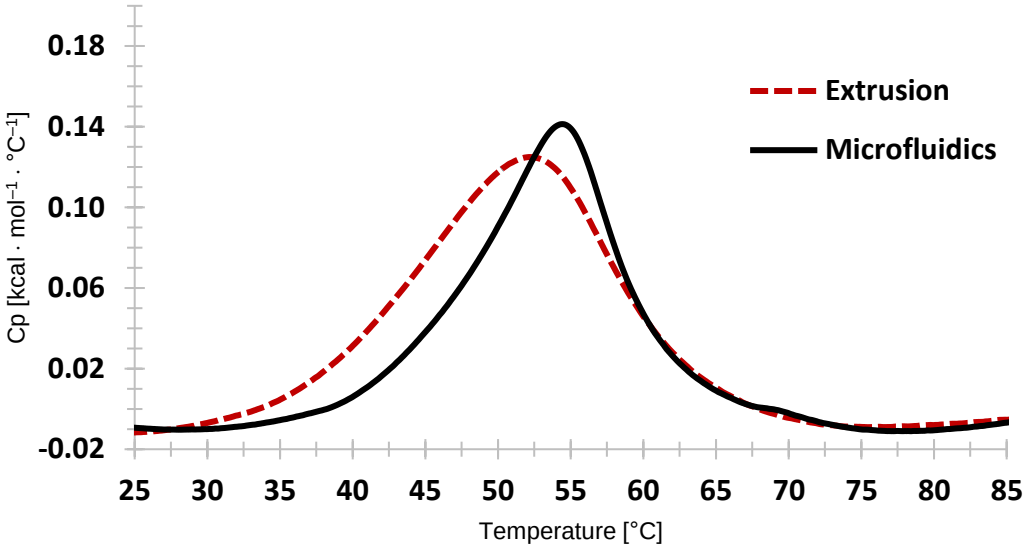


Figure 5: DSC thermograms of nanoliposomes with calcium acetate transmembrane gradient produced using a microfluidic based process and conventional extrusion.

The differences observed in the comparison should be real as the PEGylated nanoliposomes were prepared with the same lipid mix batch and based on previously proven excellent repeatability and reproducibility with less than 5% relative standard deviation (RSD) for T_m, $\Delta T_{1/2}$ and ΔH for the membrane lipid related endotherms [41]. The narrower $\Delta T_{1/2}$ reflect a more cooperative and homogeneous phase transition, because of a narrower size distribution which is indeed the case (see Table 4). Conversely, the extrusion method resulted in a broader phase transition with a slightly higher enthalpy change, potentially reflecting increased heterogeneity in lipid packing. These findings, which are consistent with size distribution results (Table 4), suggest that while both methods produce formulations with similar overall thermotropic behavior, the microfluidics approach may offer advantages in terms of uniformity.

Table 4*:* Thermodynamic parameters and size distribution of nanoliposomes with calcium acetate transmembrane gradient produced using a microfluidic based process and conventional extrusion.

Fabrication method	**T_m** [°C]	**ΔH** [kcal · mol^{-1}]	**$\Delta T_{1/2}$** [°C]	**Z-Average** [nm]	**D_i10** [nm]	**D_i50** [nm]	**Di90** [nm]	**SPAN ()**
Extrusion	52.1	2.00	14.78	72 ± 2.7	50 ± 1.1	80 ± 2.1	121 ± 4.9	0.88
Microfluidics	54.4	1.63	10.15	70 ± 0.9	51 ± 0.7	73 ± 1.2	107 ± 4.2	0.77

3.3.2 Comparison of Remotely-Loaded Continuously Manufactured Ammonium Sulfate Liposomes with Commercial Liposomal Doxorubicin

Similar to nanoliposomes passively loaded with calcium acetate, nanoliposomes passively loaded with ammonium sulfate were optimized for an active remote loading with the anticancer drug doxorubicin. The experimental investigations on the feasibility of the continuous liposomal formulation were carried out using a lab-scale set-up consisting of either a CAT300 or CAT600 micromixer (depending on TFR) and HPLC pumps as described above. Again, the commercial liposomal doxorubicin quality attributes of the target size below 100 nm average hydrodynamic diameter, PDI below 0.1 and SPAN <0.75 were met. After optimization of flow conditions during self-assembly as well as downstream processing to avoid high shear forces during the entire manufacturing process, liposomal formulations have been consistently reproduced with the same high quality at two different scales from 10 to 100 mL · min^{-1}. Conductivity and DLS measurements were performed during process optimization and finally confirmed the process stability.

We compared three different Doxil®-like drug-products: the original Caelyx® manufactured by Janssen, the FDA approved generic version of Doxil® produced by Ayana Pharma (both downsized *via* MLV extrusion) and a Doxil®-like formulation in which the PEGylated nanoliposomes were produced by microfluidics. Otherwise, all three had identical lipid composition and their lipids were hydrated by 250 mM ammonium sulfate. All further steps of the production process were identical as described elsewhere [47]. The comparison is based on the generic Doxil® major features described in the FDA Guidance [48] with focus on features obtained with the aid of cryogenic transmission electron microscopy. The Cryo-TEM images of the three Doxil®-like liposomes studied are summarized in Figure 6.

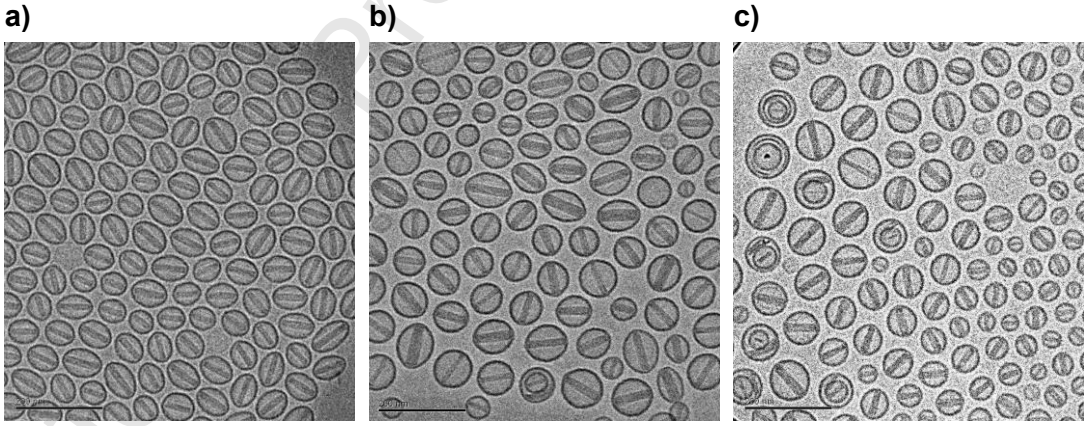


Figure 6. CryoTEM images of Doxil®-like liposomes prepared by a) Fraunhofer IMM using microfluidics, further processed, and loaded with Doxorubicin by Ayana Pharma; b) Caelyx® and c) Ayana Pharma starting with stepwise extrusion downsizing. Scalebar 200 nm.

The image analysis that was performed by the VAS software from QuTEM AB is presented in Table 5, that includes statistical data on the liposomes size, size distribution, morphology and lamellarity. The results indicate that the Doxil®-like drug-product prepared by microfluidics as the first production step exhibits the best uniformity in terms of size, size distribution, shape distribution, and the lowest percentage of non-unilamellar liposomes.

Significant differences were found in the size distribution and the axial ratio (elongatedness), the latter difference may reflect the differences in doxorubicin-sulfate nano-rod crystal length [40]. Regarding size analysis it is difficult to compare size measurements when the axial ratio (deviation from sphericity) is not the same, which is the case here. This difference indicates the necessity of more elaborated comparison of both the pharmaceutical quality (chemicals, manufacturing, and controls) and bioequivalence.

Table 5. Cryo-TEM comparison of Doxil®-like liposomes prepared with either microfluidics or extrusion. Analysis was done by measurement of multiple images with a total of N>1000 particles as representative sample according to reference [40]

	Size [nm]				SPAN	Non-Uni-Lamellar Liposomes [%]	Axis ratio
	Mean	D_i10	D_i50	D_i90			
Microfluidics	74.6	63.7	74.3	85.8	0.30	1.0	1.27
Caelyx®*	75.2	50.2	75.8	96.9	0.62	5.0	1.10
Extrusion**	63.7	47.7	61.8	83.9	0.59	8.0	1.08

*Janssen Lot LFZT800; **Ayana Lot 220123.

In addition to the morphological properties of the dissimilar manufactured liposomes, we examined their stability and ethanol content on different time scales. Both production processes endeavor to reduce the ethanol content as soon as possible after production by dialysis or TFF processing. Due to the different but initially high ethanol contents and the thermal and mechanical stress of the extruded liposomes, a comparison directly after production with a subsequent downstreaming process up to shelf-life stability of several months is meaningful. We therefore studied several batches of extrusion-based PEGylated nanoliposomes (Figure 7 a and b) with produced equivalents *via* microfluidics (Figure 8) and analyzed them comprehensively with various methods (DLS, Headspace GC, DSC). The results confirmed that the liposomes remained stable for at least 24 months, independent on the ethanol content, as there was no significant variation in both size and intraliposomal DOX content. Changes in size and drug load during 24-month storage duration showed no significance as statistically supported.

Influence of different residual ethanol contents on the long-term stability of liposomes produced by extrusion method was investigated in the ethanol content range between <96 ppm and 380 ppm (Figure 7 *a*). According to standard deviation analysis no significant changes in size and size distribution as well as on the encapsulated amount of DXR were detected. Drug content was constantly high (>97% at the beginning compared to >96% at the end of the long-term study), which indicates the overall stability of the lipidic membrane ensuring no premature drug leakage during storage (Figure 7 b).

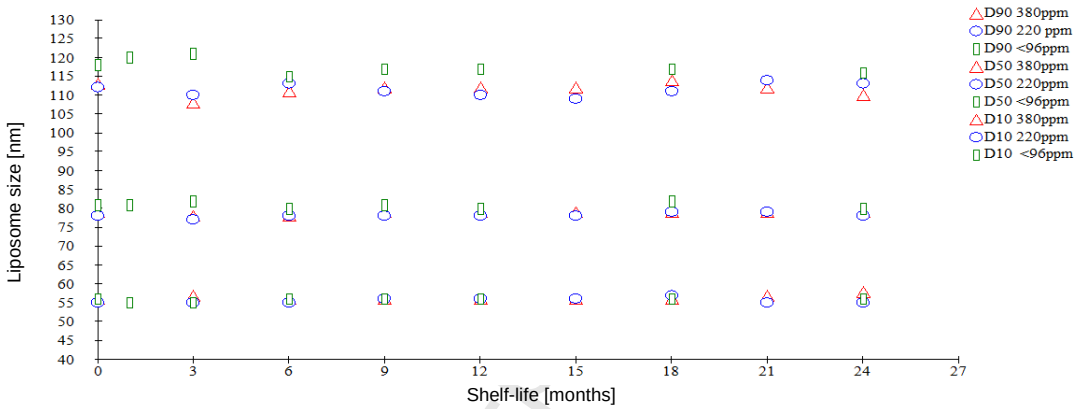


Figure 7 a. Independence of liposome size distribution from the ethanol content in the range from below 96 ppm to up to 380 ppm. These Parameters were followed up for 24 months at 5°± 3°C.

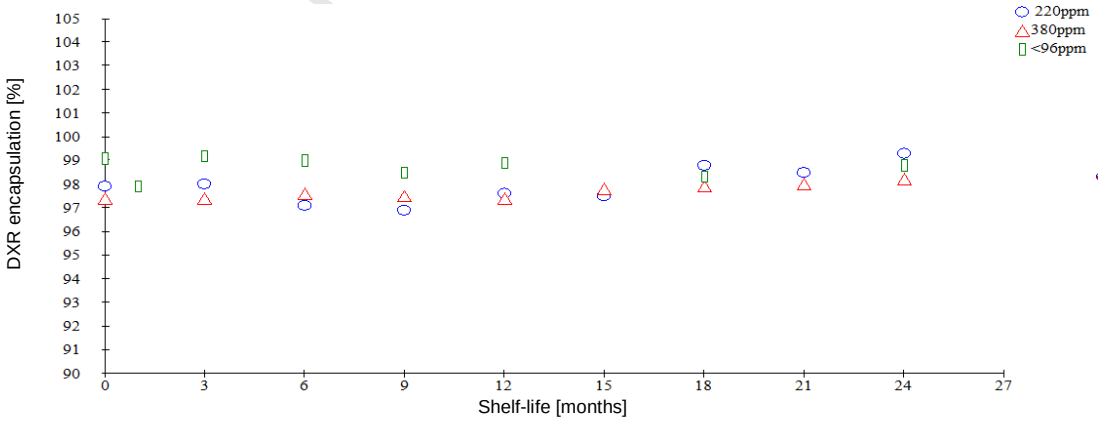


Figure 7 b. Independence of extent of doxorubicin encapsulation from ethanol content in the range from below 96 ppm to up to 380 ppm. These Parameters were followed up for 24 months at 5°± 3°C.

As can be seen in Figure 8, three batches of microfluidically produced nanoliposomes (equal ethanol content <100 ppm according to FDA requirements for pharmaceutical nanotherapeutics, analyzed by headspace GC) have also displayed long-term stability and have shown no significant changes (ANOVA, Figure 8) in size and size distribution over a period of 42 months which indicates an exceptional morphological stability in terms of hydrodynamic properties over the entire period. **Figure 7**

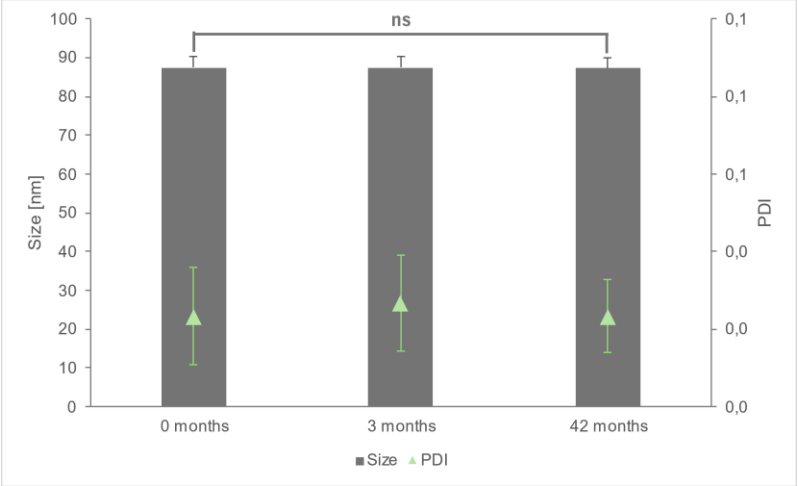


Figure 8. Long-term stability of microfluidically prepared liposomes (three individual production runs) that comply with FDA guidelines for ethanol content of <100 ppm. DLS-Data was analyzed using One way ANOVA showing no significance regarding changes in size and PDI during the entire observation period.

4. Discussion

Numerous microfluidic as well as high-pressure T-mixing (impingement jet) methods have been developed in academia and industry which were mostly designed and tested for lipid nanoparticle formulations [49]–[51]. During the Covid-19 pandemic, continuous high-pressure T-mixing manufacturing of lipid nanoparticles (LNP) enabled the huge scale production of lipid mRNA-LNP vaccines needed to immunize billions of people. As already explained in the introduction, although nanoliposomes and LNPs are both mainly based on polar lipids and are both in the nanoscale range, they differ considerably in their structure and morphology. Based on size distribution described by PDI and D_i10, D_i50, D_i90 (and their SPAN) combined with cryo-TEM, the latter mRNA-LNP vaccines exhibit a broad size distribution [52]. In addition, the shelf life of these vaccines requires storage in a frozen state (−80˚C for Comirnaty and −50 to −20˚C for Spikevax) [15],[53]. However, without investigating and comparing the commercial mRNA-LNP vaccines with the same vaccines produced by optimal microfluidics, it is difficult to say what is responsible for the defects of the vaccine envelope (that their presence may be important to the vaccine function [15]): is it the lipid composition and especially the physicochemical nature of the ionizable lipid, or the method of vaccine preparation or both?

Most of the LNP production technologies have also been evaluated for liposome production. Even though the high potential of microfluidic approaches as an alternative to extrusion-based production processes has already been discussed, there is no head-to-head comparison between nanoliposomal drug-products whose first production step is based on microfluidics and those whose first production stage is extrusion of MLVs followed by identical production steps. Namely the only difference in the drug-product production is the downsizing method. To our best knowledge currently there is no marketed nanoliposome drug-product manufactured by microfluidics [54]. Here, for the first time microfluidic manufacturing of drug-loaded nanoliposomes compared to marketed liposomal drug products as generic Doxil® have been presented in a comparable extrusion-based production scale. The micro mixer set-up can operate at more than 10 to 50 times higher flow rates than the marketed lab scale devices without parallelization. We have produced batches in the decaliter volume range, and the microfluidics device used operates at similar high lipid concentrations than the extrusion-based method. Only a few published papers report on the production of nanoliposomes by microfluidics, in a few cases also in comparison with other manufacturing processes, and on drug-containing nanoliposomes, which have not yet reached market maturity [55]–[57]. Size and size distribution measurements by DLS and cryo-TEM of Comirnaty® (LNP based vaccine) reveals rather broad size distribution in the range of 50 to 200 nm diameter having a SPAN of 1.1 compared with 0.8 for Doxil®

mainly due to the significantly larger D_i90 for Comirnaty [15]. Such broad size distribution is accepted by the FDA and EMA for vaccines but not for drug-products administered systemically. Still, continuous, industrial-scale liposome production processes that meet the quality requirements of the FDA and the EMA for systemic administered nanodrug products, and which can compete with the extrusion process are rarely reported.

In this work we have shown that it is possible to produce PEGylated nanoliposomes with scalable microfluidics in a continuous flow process below the range of lipid phase transition temperatures. Our approach combines the advantages of industrial scale-up capability (up to the hectoliter scale) and fabrication in an equipment made of stainless steel with the controlled mixing achievable with microfluidics, retaining the option to scale-up internal structures *versus* scale-out, as typically done by numbering up and parallelization in microfluidic approaches.

Currently, the industrial-scale production of most nanoliposomal drug-products is still carried out in a multi-step batch process. The first step starts with the hydration of the lipids used for liposome production above the “liposome forming lipids” (usually phosphatidylcholine, PC) phase transition temperature range to form multilamellar vesicles (MLV) [58]. The subsequent downsizing to diameters <200 nm is done either by extrusion above the phase transition of the liposome forming lipids at the desired aqueous medium to high pressure through a separation layer with an appropriately defined pore size (e.g., manufacturing Doxil®) [59]–[61] or by high-pressure homogenization (e.g., manufacturing AmbiSome® and cosmetic-relevant nanoliposomes) [62]–[64]. The reason for the need of being above phase transition temperature stems from the need of the lipids that form the liposomes to be in a fluidic state to be fully hydrated which allows intact MLVs production and provides the elasticity enabling shape changes required for the extrusion to occur [52]. One of the well-recognized issues of the extrusion process is that in many cases the pores of the filter get clogged leading to the need to stop the process, open the extruder, and change the filter (or filter “sandwich”). This slows down production and adds risk factors related to sterility.

High pressure homogenization often leads to broad size distribution including excessive formation of very small liposomes. It may also lead to high temperatures and shear forces [65]–[67]. The above differences and deficiencies of extrusion and homogenization are related to their being “top-down” processes, while the microfluidic based production is the opposite, a “bottom-up” process. Therefore, by applying the continuous micromixer-based process approach, the challenges mentioned above can be overcome. Thermosensitive lipids and even new generation drugs (e.g., nucleic acid-based immunotherapeutic) can be formulated to nanoparticles at room or even lower temperature with the platform technology presented here in a scalable and material- as well as time-saving way, while fulfilling aseptic and reproducible process control as well as GMP compliance according to FDA and EMA guidelines.

Furthermore, microfluidics based on the “bottom-up” approach starting with ethanolic lipid solution ensures improved *in situ* size control during liposome production, avoiding subsequent downsizing and homogenization steps, as well as exposures to high temperature for a significant time. This minimizes material loss and avoids the time-consuming replacement of sterile filters in aseptic extrusion processes as well as ensuring a better chemical stability of the lipids and the encapsulated drug-products.

Scale-up options are available for both mixing principles presented in this study. A caterpillar micromixer can be scaled up by increasing the internal dimensions from 150 µm up to 2400 µm. In case of the slit interdigital micromixer with 45 µm channel width for small-scale mixing, the principle of multi-lamination can be scaled-up with a so-called star-laminator mixer. In this mixer, an array of several alternating star- and circular-shaped diaphragms (foils) with defined pore sizes and spacings divides the volume flow into multiple thin lamellae (multi-lamination) in each stage, which are recombined at the end of the mixing unit and focused to a single outlet stream (Figure 9). This mixing device has already been used for an emulsion process with a throughput of up to several tons per hour [68].

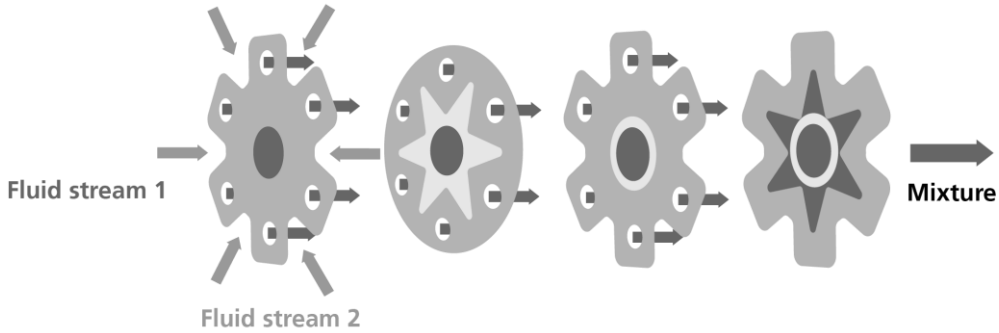


Figure 9. Assembly and mixing structure of a starlaminator micromixer: The mixer is composed of an array of alternating star-like and disk-like foils with defined pores, total stream will be focused on the middle of the device and flow out at a single outlet [68].

This continuous flow liposome production based on microfluidics is feasible for versatile liposomal drug products. Preparing liposomes exhibiting transmembrane ion gradient for subsequent remote active loading is beneficial in many aspects. They have a long shelf life if they are produced aseptically or sterile filtered. Drug loading with highly potent and toxic APIs can be performed in specially equipped facilities to handle these substances safely, or just before injection into the patient at the hospital (Myocet®) [69]. Furthermore, these transmembrane ions gradient exhibiting nanoliposomes can be commercially ordered to study drug loading and drug release profiles for the development of new nanopharmaceuticals [70].
Other microreactor-based formulation processes have also demonstrated impressive results regarding size control and rather narrow size distribution, however the proof of successful stable remote loading with relevant drugs to compete with marketed products which is the best indication to liposome membrane integrity was still missing [71],[72] until this study. Instruments for microfluidic formulation have become state of the art and first publications have arisen almost 10 years ago. Academic investigations on encapsulation of poorly water-soluble drugs as well as dual loading hydrophilic and hydrophobic drugs have also been published already [73],[74]. Process volume of research instruments are generally low and scale-up of microfluidics was for a long period only feasible by parallelization [75].Recently, more frequently larger scale and further development towards GMP manufacturing became commercially available. However, decaliter scale to produce nanosized liposomes via microfluidic mixing without parallelization has not yet commercialized to our knowledge. Due to the special design of its microfluidic mixing chamber, the CAT micromixer used here is able to scale up the throughput with increasing in inner dimension of the microfluidic structures [42].

This study shows that nanoliposomal drug-products with critical quality characteristics concerning size and size distribution, and other features required by FDA and EMA [76] (as for liposomal doxorubicin) prepared by large scale continuous microfluidics-based processes can compete with similar liposomal drug-product prepared by the non-continuous extrusion process.

5. Conclusions

Our studies demonstrate the following advantages of the continuous manufacturing based on micro mixing approach:

- This study shows that PEGylated nanoliposomes remotely loaded with either amphipathic weak bases or acids, exhibit *in vitro* bioequivalency in all physicochemical attributes as defined by the FDA guidance on generic Doxil®[48]. In terms of size distribution and uniformity the nanoliposomes prepared via microfluidics is narrower compared to the same liposomal nanodrugs produced by the conventional extrusion-based method of lipid hydration to form MLVs followed by down-sizing. These findings are consistent with DSC results, demonstrating that PEGylated nanoliposomes produced

by microfluidics exhibit similar thermotropic behavior while the exact DSC results support their higher uniformity and greater cooperativity in the lipid bilayer membrane.

- Being a “bottom-up” process, continuous manufacturing based on micro mixing approach does not require working at a temperature above the lipid phase transition temperature, while production based on extrusion being a “top-down” process has an absolute requirement to perform the production above the lipid phase transition temperature. Thereby, potential degradation of lipids and drug-substances is minimized.
- Our approach combines the advantages of industrial scale-up capability (up to the hectoliter scale) and fabrication in a stainless-steel set-up with the controlled mixing that can be achieved with microfluidics, retaining the option of linear scale-up through internal structures versus scale-out, as typically done by numbering up and parallelization in microfluidic approaches.

6. Patent applications and patents

Regina Bleul and Raphael Thiermann: “Method and device for producing a liquid containing liposomes, and produced liquid”, PCT/EP2021/082320, DE, EP, CN, IL and US Patent Applications, November 19, 2019.

Yechezkel Barenholz and Gilad Haran: “Method of Amphipathic Drug Loading in Liposomes by pH Gradient”. U.S. Patent No. 5192549, March 9, 1993.

Yechezkel Barenholz and Gilad Haran: “Liposomes: Efficient Loading and Controlled Release of Amphipathic Molecules” “Method for amphipathic drug loading in liposomes by ammonium ion gradient”. U.S. Patent 5316771, May 31, 1994.

Yechezkel Barenholz, Alberto A. Gabizon and Yuval Avnir. “Liposomal Compositions of Glucocorticoid and Glucocorticoid Derivatives”. US Patent 8932627, January 13, 2015.

Author Contributions

A.-N E.-K., K.T., D.S, C.P., R.T. performed the synthesis and characterization of the samples. D.S. and R.T. performed cryogenic transmission electron microscopy measurements. A.-N E.-K., K.T., D.S, R.B. wrote the manuscript; R.B., Y.B. reviewed and edited the manuscript. Work was supervised by R.B. (microfluidics) and Y.B. (clinically relevant products); project administration: R.B., K.T. and Y.B.; funding acquisition: R.B., Y.B. All authors have read and agreed to the published version of the manuscript.

Funding

This work was supported by the Federal Ministry of Education and Research (BMBF) [Grant no. 13XP5113]; the German Research Foundation within the collaborative research center [SFB 1552/1, no 465145163, project C01]; and the Barenholz Fund, which was established with a part of the royalties from Barenholz and is used to support research in the Barenholz laboratory.

Competing financial interests' statement

Regina Bleul and Raphael Thiermann are the inventors of the yet not licensed patent application “Method and device for producing a liquid containing liposomes and —produced liquid” (PCT/EP2021/082320, DE, EP, CN, IL and US Patent Applications, November 19, 2019) which covers the continuous process used here for preparing nanoliposomal dispersions by micromixers.
Yechezkel Barenholz is one of the inventors of two already-expired (March 2010) patents relevant to Doxil®: (1) Barenholz, Y., and Haran, G. “Method of Amphipathic Drug Loading in Liposomes by pH Gradient” (U.S. Patent 5192549, March 9, 1993. U.S. Patent 5244574, September 14, 1993); (2) Barenholz Y., and Haran, G. “Liposomes: Efficient Loading and Controlled Release of Amphipathic Molecules” (U.S. Patent 5316771, May 31, 1994). The Hebrew University received royalties from Doxil® sales until the patents expired. In addition, Barenholz is one of the inventors of “Liposomal Compositions of Glucocorticoid and Glucocorticoid Derivatives” (Yechezkel Barenholz, Alberto A. Gabizon and Yuval Avnir, US Patent 8932627, January 13, 2015) owned by Yissum LTD of the TTOs of the Hebrew University of Jerusalem. Yechezkel Barenhoz is also the CSO of Lipocure Ltd., a start-up that develops liposomal drugs.

Acknowledgments
We thank Lipoid GmbH for supplying us the ready to use Lipid-Mix for the nanoliposomes loaded with MPS. Doxil® like pegylated nanoliposomes were also prepared with the same Lipoid Lipid-Mix. Many thanks to Nadja Hellmann (Department of Chemistry/Biochemistry, Johannes-Gutenberg-University Mainz) and Nils Meier (Fraunhofer IMM Nanomedicine Team) for their leading support in the differential thermal analysis of liposomes. Thanks also to Eilien Heinrich (Fraunhofer IMM Nanomedicine Team) for the statistical evaluation of the test batches using ANOVA tests. We also thank Gabriele Menges-Flanagan (Fraunhofer IMM) for her help in editing the English version of the manuscript.

References

[1] Bozzuto, G.; Molinari, A. Liposomes as nanomedical devices. *Int. J. Nanomedicine* **2015**, *10*, 975–999. doi: 10.2147/IJN.S68861.

[2] Rodríguez, F.; Caruana, P.; De la Fuente, N.; Español, P.; Gámez, M.; Balart, J.; Llurba, E.; Rovira, R.; Ruiz, R.; Martín-Lorente, C.; Corchero, J.L.; Céspedes, M.V. Nano-Based Approved Pharmaceuticals for Cancer Treatment: Present and Future Challenges. *Biomolecules* **2022**, *12* (6), 784. doi: 10.3390/biom12060784.

[3] Giordani, S.; Marassi, V.; Zattoni, A.; Roda, B.; Reschiglian, P. Liposomes characterization for market approval as pharmaceutical products: Analytical methods, guidelines and standardized protocols. *J. Pharm. Biomed. Anal.* **2023**, *236*, 115751. doi: 10.1016/j.jpba.2023.115751.

[4] Smith, J.A.; Costales, A.B.; Jaffari, M.; Urbauer, D.L.; Frumovitz, M.; Kutac, C.K.; Tran, H.; Coleman, R.L. Is it equivalent? Evaluation of the clinical activity of single agent Lipodox® compared to single agent Doxil® in ovarian cancer treatment. *J. Oncol. Pharm. Pract.* **2016**, *22* (4), 599–604. doi: 10.1177/1078155215594415.

[5] Wang, S.; Chen, Y.; Guo, J.; Huang, Q. Liposomes for Tumor Targeted Therapy: A Review. *Int. J. Mol. Sci.* **2023**, *24*, 2643. doi: 10.3390/ijms24032643.

[6] van Alem, C.M.A.; Metselaar, J.M.; van Kooten, C.; Rotmans, J.I. Recent Advances in Liposomal-Based Anti-Inflammatory Therapy. *Pharmaceutics* **2021**, *13*, 1004. doi: 10.3390/pharmaceutics13071004.

[7] Adler-Moore, J.; Proffitt, R.T. AmBisome: liposomal formulation, structure, mechanism of action and pre-clinical experience. *J. Antimicrob. Chemother.* **2002**, *49*, 21–30. doi: 10.1093/jac/49.suppl_1.21.

[8] Kaye, A.D.; Armstead-Williams, C.; Hyatali, F.; Cox, K.S.; Kaye, R.J.; Eng, L.K.; Faroog Anwar, M.A.; Patel, P.V.; Patil, S.; Cornett, E.M. Exparel for Postoperative Pain Management: a Comprehensive Review. *Curr. Pain Headache Rep.* **2020**, *24*, 73. doi: 10.1007/s11916-020-00905-4.

[9] Barenholz, Y. Doxil® — The first FDA-approved nano-drug: Lessons learned. *J. Control. Release* **2012**, *160* (2), 117–134. doi: 10.1016/j.jconrel.2012.03.020.

[10] Peng, T.; Xu, W.; Li, Q.; Ding, Y.; Huang, Y. Pharmaceutical liposomal delivery—specific considerations of innovation and challenges. *Biomater. Sci.* **2023**, *11*, 62. doi: 10.1039/d2bm01252a.

[11] Weng, Y.; Li, C.; Yang, T.; Hu, B.; Zhang, M.; Guo, S.; Xiao, H.; Liang, X.J.; Huang, Y. The challenge and prospect of mRNA therapeutics landscape. *Biotechnol. Adv.* **2020**, *40*, 107534. doi: 10.1016/j.biotechadv.2020.107534.

[12] Urits, I., Swanson, D., Swett, M.C.; Patel, A.; Berardino, K.; Amgalan, A.; Berger, A.A.; Kassem, H.; Kaye, A.D.; Viswanath, O. A Review of Patisiran (ONPATTRO®) for the Treatment of Polyneuropathy in People with Hereditary Transthyretin Amyloidosis. *Neurol. Ther*. **2020**, *9*, 301–315. doi: 10.1007/s40120-020-00208-1.

[13] Breakthrough technologies across different drug classes to revolutionize medicine. Available online: https://www.biontech.com/int/en/home/pipeline-and-products/pipeline.html (accessed on 30 September 2024).

[14] Barenholz, Y. Liposome application: problems and prospects. *Curr. Opin. Colloid Interface Sci.* **2001**, *6* (1), 66–77. doi: 10.1016/S1359-0294(00)00090-X.

[15] Szebeni, J.; Kiss, B.; Bozó, T.; Turjeman, K.; Levi-Kalisman, Y.; Barenholz, Y.; Kellermayer, M. Insights into the Structure of Comirnaty Covid-19 Vaccine: A Theory on Soft, Partially Bilayer-Covered Nanoparticles with Hydrogen Bond-Stabilized mRNA–Lipid Complexes. *ACS Nano* **2023**, *17* (14), 13147–13157. doi: 10.1021/acsnano.2c11904.

[16] Web of Science Topic Search. Available online: https://www.webofscience.com/wos/woscc/basic-search (accessed on 30 September 2024).

[17] Dadpour, S.; Mehrabian, A.; Arabsalmani, M.; Mirhadi, E.; Askarizadeh, A.; Mashreghi, M.; Jaafari, M.R. The role of size in PEGylated liposomal doxorubicin biodistribution and anti-tumour activity. *IET Nanobiotechnology* **2022**, *16* (7–8), 259–272. doi: 10.1049/nbt2.12094.

[18] Nsairat, H.; Khater, D.; Sayed, U.; Odeh, F.; Al Bawab, A.; Alshaer, W. Liposomes: Structure, composition, types, and clinical applications. *Heliyon* **2022**, *8* (5), e09394. doi: 10.1016/j.heliyon.2022.e09394.

[19] Toh, M.-R.; Chiu, G.N.C. Liposomes as sterile preparations and limitations of sterilisation techniques in liposomal manufacturing. *Asian J. Pharm. Sci.* **2013**, *8* (2), 88–95. doi: 10.1016/j.ajps.2013.07.011.

[20] Safra, T.; Muggia, F.; Jeffers, S.; Tsao-Wei, D.D.; Groshen, S.; Lyass, O.; Henderson, R.; Berry, G.; Gabizon, A. Pegylated liposomal doxorubicin (doxil): reduced clinical cardiotoxicity in patients reaching or exceeding cumulative doses of 500 mg/m². *Annals of Oncology* **2000**, *11* (8), 1029–1033. doi: 10.1023/a:1008365716693.

[21] Judson, I.; Radford, J.A.; Harris, M.; Blay, J.-Y.; van Hoesel, Q.; le Cesne, A.; van Oosterom, A.T.; Clemons, M.J.; Kamby, C.; Hermans, C.; Whittaker, J.; Donato die Paola, E.; Verweij, J.; Nielsen, S. Randomised phase II trial of pegylated liposomal doxorubicin (DOXIL®/CAELYX®) versus doxorubicin in the treatment of advanced or metastatic soft tissue sarcoma: a study by the EORTC Soft Tissue and Bone Sarcoma Group. *Eur. J. Cancer* **2001**, *37*, 870–877. doi: 10.1016/S0959-8049(01)00050-8.

[22] Pautier, P.; Italiano, I.; Piperno-Neumann, S.; Chevreau, C.; Penel, N.; Firmin, N.; Boudou-Rouquette, P.; Bertucci, F.; Balleyguier, C.; Lebrun-Ly, V.; Ray-Coquard, I.; Kalbacher, E.; Bardet, A.; Bompas, E.; Collard, O.; Isambert, N.; Guillemet, C.; Rios, M.; Archambaud, B.; Duffaud, F. Doxorubicin alone versus doxorubicin with trabectedin followed by trabectedin alone as first-line therapy for metastatic or unresectable leiomyosarcoma (LMS-04): a randomised, multicentre, open-label phase 3 trial. *Lancet Oncol.* **2022**, *23* (8), 1044–1054. doi: 10.1016/S1470-2045(22)00380-1.

[23] Rafiyath, S.M.; Rasul, M.; Lee, B.; Wie, G.; Lamba, G.; Liu, D. Comparison of safety and toxicity of liposomal doxorubicin vs. conventional anthracyclines: a meta-analysis. *Exp. Hematol. Oncol.* **2012**, *1* (10). doi: 10.1186/2162-3619-1-10.

[24] von Bomhard, S.; Schramm, J.; Bleul, R.; Thiermann, R.; Höbel, P.; Krtschil, U.; Löb, P.; Maskos, M. Modular Manufacturing Platform for Continuous Synthesis and Analysis of Versatile Nanomaterials. *Chem. Eng. Technol*. **2019**, *42* (10), 2085–2094. doi: 10.1002/ceat.201900115.

[25] Thiermann, R.; Bleul, R.; Maskos, M. Kinetic Control of Block Copolymer Self-Assembly in a Micromixing Device – Mechanistical Insight into Vesicle Formation Process. *Macromol. Chem. Phys.* **2017**, *218*, 1600347. doi: 10.1002/macp.201600347.

[26] Bauer, T.A.; Schramm, J.; Fenaroli, F.; Siemer, S.; Seidl, C.I.; Rosenauer, C.; Bleul, R.; Stauber, R.H.; Koynov, K.; Maskos, M.; Barz, M. Complex Structures Made Simple – Continuous Flow Production of Core Cross-Linked Polymeric Micelles for Paclitaxel Pro-Drug-Delivery. *Adv. Mater.* **2023**, *35* (21), 2210704. doi: 10.1002/adma.202210704.

[27] Xu, R.; Tomeh, M.A.; Ye, S.; Zhang, P.; Lv, S.; You, R.; Wang, N.; Zhao, X. Novel microfluidic swirl mixers for scalable formulation of curcumin loaded liposomes for cancer therapy. *Int. J. Pharm*. **2022**, *622*, 121857. doi: 10.1016/j.ijpharm.2022.121857.

[28] Tran, R.; Ho, S.; Dea, P. Effects of ethanol on lipid bilayers with and without cholesterol: the distearoylphosphatidylcholine system. *Biophys. Chem.* **2004**, *110* (1–2), 39–47. doi: 10.1016/j.bpc.2004.01.005.

[29] Avnir, Y.; Ulmansky, R.; Wasserman, V.; Even-Chen, S.; Broyer, M.; Barenholz, Y.; Naparstek, Y. Amphipathic weak acid glucocorticoid prodrugs remote-loaded into sterically stabilized nanoliposomes evaluated in arthritic rats and in a Beagle dog: A novel approach to treating autoimmune arthritis. *Arthritis Rheum* **2008**, *58* (1), 119–129. doi: 10.1002/art.23230.

[30] Bavli, Y.; Chen, B.-M.; Roffler, S.R.; Dobrovolskaia, M.A.; Elnekave, E.; Ash, S.; Barenholz, Y.; Turjeman, K. PEGylated Liposomal Methyl Prednisolone Succinate does not Induce Infusion Reactions in Patients: A Correlation Between in Vitro Immunological and in Vivo Clinical Studies. *Molecules* **2020**; *25* (3), 558. doi: 10.3390/molecules25030558.

[31] Gabizon, A.; Shmeeda, H.; Barenholz, Y. Pharmacokinetics of Pegylated Liposomal Doxorubicin: Review of Animal and Human Studies. *Clin. Pharmacokinet.* **2003**; *42* (5), 419–436. doi: 10.2165/00003088-200342050-00002.

[32] Al-Nadaf, S.; Rebhun, R.B.; Curran, K.M.; Venable, R.O.; Skorupski, K.A.; Willcox, J.L.; Burton, J.H. Retrospective analysis of doxorubicin and prednisone as first-line therapy for canine B-cell lymphoma. *BMC Vet. Res.* **2018**, *14* (1), 356. doi: 10.1186/s12917-018-1688-5.

[33] Schilt, Y.; Berman, T.; Wei, X.; Barenholz, Y.; Raviv, U. Using solution X-ray scattering to determine the high-resolution structure and morphology of PEGylated liposomal doxorubicin nanodrugs. *Biochim. Biophys. Acta* **2016**, *1860* (1 Pt A), 108–119. doi: 10.1016/j.bbagen.2015.09.012.

[34] Haran, G.; Cohen, R.; Bar, L.K.; Barenholz, Y. Transmembrane ammonium sulfate gradients in liposomes produce efficient and stable entrapment of amphiphatic weak bases. *Biochim. Biophys. Acta* **1993**, *1151* (2), 201–215. doi: 10.1016/0005-2736(93)90105-9.

[35] Clerc, S.; Barenholz, Y. Loading of amphipathic weak acids into liposomes in response to transmembrane calcium acetate gradients. *Biochim. Biophys. Acta* **1995**, *1240* (2), 257–265. doi: 10.1016/0005-2736(95)00214-6.

[36] Roces, C.B.; Kastner, E.; Stone, P.; Lowry, D.; Perrie, Y. Rapid Quantification and Validation of Lipid Concentrations within Liposomes. *Pharmaceutics* **2016**, *8* (3), 29. doi: 10.3390/pharmaceutics8030029.

[37] Amselem, S.; Cohen, R.; Druckmann, S.; Gabizon, A.; Goren, D.; Abra, R.M.; Huang, A.; New, R.; Barenholz, Y. Preparation and Characterization of Liposomal Doxorubicin for Human Use. *J. Liposome Res.* **1992,** *2* (1), 93–123. doi: 10.3109/08982109209039903.

[38] Anderson, B.D.; Taphouse, V. Initial Rate Studies of Hydrolysis and Acyl Migration in Methylprednisolone 21-Hemisuccinate and 17-Hemisuccinate. *J. Pharm. Sci.* **1981**, *70* (2), 181–186. doi: 10.1002/jps.2600700217.

[39] Elizondo, E.; Moreno, E.; Cabrera, I.; Córdoba, A.; Sala, S.; Veciana, J.; Ventosa, N. Liposomes and Other Vesicular Systems: Structural Characteristics, Methods of Preparation, and Use in Nanomedicine. *Prog Mol. Biol. Transl. Sci.* **2011**, *104*, 1–52. doi: 10.1016/B978-0-12-416020-0.00001-2.

[40] Nordström, R.; Zhu, L.; Härmark, J.; Levi-Kalisman, Y.; Koren, E.; Barenholz, Y.; Levinton, G.; Shamrakov, D. Quantitative Cryo-TEM Reveals New Structural Details of Doxil-Like PEGylated Liposomal Doxorubicin Formulation. *Pharmaceutics* **2021**, *13* (1), 123. doi: 10.3390/pharmaceutics13010123.

[41] Wei, X.; Cohen, R.; Barenholz, Y. Insights into composition/structure/function relationships of Doxil® gained from "high-sensitivity" differential scanning calorimetry. *Eur. J. Pharm. Biopharm.* **2016**, *104*, 260–270. doi: 10.1016/j.ejpb.2016.04.011.

[42] Ziegenbalg, D.; Kompter, C.; Schönfeld, F.; Kralisch, D. Evaluation of different micromixers by CFD simulations for the anionic polymerization of styrene. *Green Process Synth* **(2012)**, *1* (2*),* 211–224. doi: 10.1515/gps-2012-0004.

[43] Turjeman, K.; Bavli, Y.; Kizelsztein, P.; Schilt, Y.; Allon, N.; Blumenfeld Katzir, T.; Sasson, E.; Raviv, U.; Ovadia, H.; Barenholz, Y. Nano-Drugs Based on Nano Sterically Stabilized Liposomes for the Treatment of Inflammatory Neurodegenerative Diseases. *PLoS ONE* **2015**, *10* (7), e0130442. doi: 10.1371/journal.pone.0130442.

[44] Ulmansky, R.; Turjeman, K.; Baru, M.; Katzavian, G.; Harel, M.; Sigal, A.; Naparstek, Y.; Barenholz, Y. Glucocorticoids in nano-liposomes administered intravenously and subcutaneously to adjuvant arthritis rats are superior to the free drugs in suppressing arthritis and inflammatory cytokines. *J. Control. Release* **2012**, *160* (2), 299–305. doi: 10.1016/j.jconrel.2011.12.024.

[45] Garbuzenko, O.; Zalipsky, S.; Qazen, M.; Barenholz, Y. Electrostatics of PEGylated micelles and liposomes containing charged and neutral lipopolymers. *Langmuir* **2005**, *21*, 2560–2568. doi: 10.1021/la0479105.

[46] Avnir, Y.; Turjeman, K.; Tulchinsky, D.; Sigal, A.; Kizelsztein, P.; Tzemach, D.; Gabizon, A.; Barenholz, Y. Fabrication Principles and Their Contribution to the Superior In Vivo Therapeutic Efficacy of Nano-Liposomes Remote Loaded with Glucocorticoids. *PLoS ONE* **2012**, *6* (10), e25721. doi: 10.1371/journal.pone.0025721.

[47] Schilt, Y.; Berman, T.; Wei, X.; Nativ-Roth, E.; Barenholz, Y.; Raviv, U., Effect of the ammonium salt anion on the structure of doxorubicin complex and PEGylated liposomal doxorubicin nanodrugs. *Biochim. Biophys. Acta (BBA – General Subjects)* **2020**, *1865* (5), 129849. doi: 10.1016/j.bbagen.2021.129849.

[48] Draft Guidance on Doxorubicin Hydrochloride (Recommended May 2022). Available online: https://www.accessdata.fda.gov/drugsatfda_docs/psg/PSG_050718.pdf (accessed on 30 September 2024).

[49] Maeki, M.; Uno, S.; Niwa, A.; Okada, Y.; Tokeshi, M. Microfluidic technologies and devices for lipid nanoparticle-based RNA delivery. *J. Control. Release* **2022**, *344*, 80–96. doi: 10.1016/j.jconrel.2022.02.017.

[50] Carvalho, L.; Ceccato, B.T.; Michelon, M.; Han, S.W.; de la Torre, L.G. Advanced Microfluidic Technologies for Lipid Nano-Microsystems from Synthesis to Biological Application. *Pharmaceutics* **2022**, *14*(1), 141. doi: 10.3390/pharmaceutics14010141.

[51] Mehraji, S.; DeVoe, D.L. Microfluidic synthesis of lipid-based nanoparticles for drug delivery: recent advances and opportunities. *Lab Chip* **2024**, *24*, 1154–1174. doi: 10.1039/d3lc00821e.

[52] Lichtenberg, D.; Barenholz, Y. Liposomes: Preparation, characterization, and preservation. In: *Methods of Biochemical Analysis*, Glick, D. ed., Wiley, New-York, **33**, 337–462, **1988**. doi: 10.1002/9780470110546.ch7.

[53] Spikevax: EPAR - Product information. Available online: https://www.ema.europa.eu/en/documents/product-information/spikevax-epar-product-information_en.pdf (accessed on 30 September 2024).

[54] Shah, V.M.; Nguyen, D.X.; Patel, P.; Cote, B.; Al-Fatease, A.; Pham, Y.; Huynh, M.G.; Woo, Y.; Alani, A.W.G. Liposomes produced by microfluidics and extrusion: A comparison for scale-up purposes. *Nanomed.: Nanotechnol. Biol. Med.* **2019**, *18*, 146–156. doi: 10.1016/j.nano.2019.02.019.

[55] Lee, C.-Y.; Tsai, T.; Peng, P.-C.; Chen, C.-T. Fabrication of Doxorubicin-Loaded Lipid-Based Nanocarriers by Microfluidic Rapid Mixing. *Biomedicines* **2022**, *10*(6)*,* 1259*.* doi: 10.3390/biomedicines10061259.

[56] Al-Amin, M.D.; Bellato, F.; Mastrotto, F.; Garofalo, M.; Malfanti, A.; Salmaso, S. Dexamethasone Loaded Liposomes by Thin-Film Hydration and Microfluidic Procedures: Formulation Challenges. *Int. J. Mol. Sci.* **2020**, *21*(5), 1611. doi: 10.3390/ijms21051611.

[57] Levy, E.S.; Yu, J.; Estevez, A.; Mao, J.; Liu, L.; Torres, E.; Leung, D.; Yen, C.-W. A Systematic Approach for Liposome and Lipodisk Preclinical Formulation Development by Microfluidic Technology. *AAPS J.* **2021**, *23*(6), 111. doi: 10.1208/s12248-021-00651-4.

[58] Elizondo, E.; Moreno, E.; Cabrera, I.; Córdoba, A.; Sala, S.; Veciana, J.; Ventosa, N. Liposomes and Other Vesicular Systems: Structural Characteristics, Methods of Preparation, and Use in Nanomedicine. Prog *Mol. Biol. Transl. Sci.* **2011**, *104*, 1–52. doi: 10.1016/B978-0-12-416020-0.00001-2.

[59] Hope, M.J.; Bally, M.B.; Webb, G.; Cullis, P.R. Production of large unilamellar vesicles by a rapid extrusion procedure—characterization of size distribution, trapped volume and ability to maintain a membrane-potential. *Biochim. Biophys. Acta* **1985**, *812*, 55–65. doi: 10.1016/0005-2736(85)90521-8.

[60] Berger, N.; Sachse, A.; Bender, J.; Schubert, R.; Brandl, M. Filter extrusion of liposomes using different devices: comparison of liposome size, encapsulation efficiency, and process characteristics. *Int. J. Pharm.* **2001**, *223*, 55–68. doi: 10.1016/s0378-5173(01)00721-9.

[61] Shepherd, S.J.; Issadore, D.; Mitchell, M.J. Microfluidic formulation of nanoparticles for biomedical applications. *Biomaterials* **2021,** *274*, 120826. doi: 10.1016/j.biomaterials.2021.120826.

[62] Alavi, M., Mozafari, M., Hamblin, M., Hamidi, M., Hajimolaali, M., Katouzian, I. Industrial-scale methods for the manufacture of liposomes and nanoliposomes: pharmaceutical, cosmetic, and nutraceutical aspects. *Micro Nano Bio Asp.* **2022**, *1* (2), 26–35. doi: 10.22034/mnba.2022.159371.

[63] Pupo, E.; Padrón, A.; Santana, E.; Sotolongo, J.; Quintana, D.; Dueñas, S.; Duarte, C.; de la Rosa, M.C.; Hardy, E. Preparation of plasmid DNA-containing liposomes using a high-pressure homogenization-extrusion technique. *J. Control. Release.* **2005**, *104*, 379–396. doi: 10.1016/j.jconrel.2005.02.001.

[64] Vinchhi, P.; Patel, J.K.; Patel, M.M. High-Pressure Homogenization Techniques for Nanoparticles. In: *Emerging Technologies for Nanoparticle Manufacturing*, 1st ed.; Patel, J.K., Pathak, Y.V. (Eds.); Springer, Cham, 263–285, **2021**. doi: 10.1007/978-3-030-50703-9_11.

[65] Floury, J.; Desrumaux, A.; Lardières, J. Effect of high-pressure homogenization on droplet size distributions and rheological properties of model oil-in-water emulsions. *Innov. Food Sci. Emerg. Technol.* **2000**, *1* (2), 127–134. doi: 10.1016/S1466-8564(00)00012-6.

[66] Talsma, H.; Özer, A.Y.; van Bloois, L.; Crommelin, D.J.A. The Size Reduction of Liposomes with a High Pressure Homogenizer (Microfluidizer™). Characterization of Prepared Dispersions and Comparison with Conventional Methods. *Drug. Dev. Ind. Pharm.* **1989**, *15* (2), 197–207. doi: 10.3109/03639048909040205.

[67] Wang, Y.; Pisapati, A.V.; Zhang, X.F.; Cheng, X. Recent Developments in Nanomaterial-Based Shear-Sensitive Drug Delivery Systems. *Adv. Healthc. Mater*. **2021**, *10* (13), e2002196. doi: 10.1002/adhm.202002196.

[68] Werner, B.; Hessel, V.; Löb, P. Mixers with Microstructured Foils for Chemical Production Purposes. *Chem. Eng. Technol.* **2005**, *28* (4), 401–407. doi: 10.1002/ceat.200407163.

[69] European Medicines Agency, Product Information of Myocet® liposomal. Available online: https://www.ema.europa.eu/en/documents/product-information/myocet-liposomal-previously-myocet-epar-product-information_en.pdf (accessed on 30 September 2024).

[70] Tribioscience Product Portfolio. Available online: https://tribioscience.com/product/life-sciences/liposomes/hspc-chol-mpeg2000-dspe-50455-mol-mol-liposomes-with-ammonium-gradient-100nm/ (accessed on 30 September 2024).

[71] López, R.R.; Ocampo, I.; Font de Rubinat, P.G.; Sánchez, L.-M.; Alazzam, A.; Tsering, T.; Bergeron, K.-F.; Camacho-Léon, S.; Burnier, J.V.; Mounier, C.; Stiharu, I.; Nerguizian, V. Parametric Study of the Factors Influencing Liposome Physicochemical Characteristics in a Periodic Disturbance Mixer. *Langmuir* **2021**, *37* (28), 8544–8556. doi: 10.1021/acs.langmuir.1c01005.

[72] Peng, C.; Zhu, X.; Zhang, J.; Zhao, W.; Jia, W.; Wu, Z.; Yu, Z., Dong, Z. Antisolvent fabrication of monodisperse liposomes using novel ultrasonic microreactors: Process optimization, performance comparison and intensification effect. *Ultrason. Sonochem*. **2024**, *103*, 106769. doi: 10.1016/j.ultsonch.2024.106769.

[73] Kastner, E.; Verma, V.; Lowry, D.; Perrie, Y. Microfluidic-controlled manufacture of liposomes for the solubilisation of a poorly water soluble drug. *Int. J. Pharm*. **2015**, *485* (1–2), 122–130. doi: 10.1016/j.ijpharm.2015.02.063.

[74] Joshi, S.; Hussain, M.T.; Roces, C.B.; Anderluzzi, G.; Kastner, E.; Salmaso, S.; Kirby, D.; Lowry, D.; Perrie, Y. Microfluidics based manufacture of liposomes simultaneously entrapping hydrophilic and lipophilic drugs. *Int. J. Pharm*. **2016**, *514* (1), 160–168. doi: 10.1016/j.ijpharm.2016.09.027.

[75] Gkionis, L.; Aojula, H.; Harris, L.K.; Tirella, A. Microfluidic-assisted fabrication of phosphatidylcholine-based liposomes for controlled drug delivery of chemotherapeutics. *Int. J. Pharm*. **2021**, *604*, 120711. doi: 10.1016/j.ijpharm.2021.120711.

[76] FDA/CDER Guidance Document for Industry: Liposome Drug Products: Chemistry, Manufacturing, and Controls; Human Pharmacokinetics and Bioavailability; and Labeling Documentation (April 2018). Available online: https://www.fda.gov/media/70837/download (accessed on 30 September 2024).

Graphical abstract