

Manufacturing of liposomes:

A direct comparison of extrusion and microfluidics protocols

Mahmoud Tarek Sanad, Claudio Alter, Pascal Detampel, Tomaz Einfalt,

*Jörg Huwyler**

Division of Pharmaceutical Technology, Department of Pharmaceutical Sciences, University of
Basel, Switzerland

*Corresponding author: ✉ joerg.huwyler@unibas.ch

Abstract

Liposomal formulations are frequently used for oral, topical, or parenteral drug administration. However, liposome manufacturing and industrial scale-up remains a challenge, in particular if it comes to the preparation of liposome populations with a homogenous size distribution. Therefore, extrusion through filter membranes with defined pore size is traditionally used during the preparation of small unilamellar liposomes. Microfluidics is considered to be an alternative manufacturing method. Lipids, solvents and excipients are thereby passively mixed using a microfluidics device. While the microfluidic approach is highly scalable, most of the traditional liposome preparation protocols rely on extrusion. It was therefore the aim of the present study to compare liposomal formulations with identical composition, which were prepared using either extrusion or microfluidics protocols. Liposomal formulations produced by both methods were analyzed using dynamic light scattering (DLS) to compare size, polydispersity, and ζ -potential. Our results indicate significant differences between liposomal preparations obtained using the two manufacturing methods. We conclude that the two preparation methods should not be used interchangeably.

Introduction

Liposomes are used in pharmaceutical technology as nanocarriers for a broad range of compounds including antibiotics or anti-cancer drugs¹. Many liposomal formulations have been approved by regulatory authorities and are therefore part of commercial drug products¹.

The main constituents of liposomes are natural or (semi)synthetic phospholipids such as phosphatidylcholine (e.g. DOPC), 1,2-Dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE), and phosphatidylserine (PtdSer). Additional components include cholesterol and its derivatives^{2,3}. Phosphatidylserine (PtdSer) was included in the present study since it is exposed on the outer leaflet of apoptotic bodies or exosomes⁴.

Natural (phospho)lipids spontaneously form membrane bilayer structures in an aqueous solution. During formation, energy can be applied in the form of, for example, direct heating, shaking, sonication, mechanical stress⁵ in order to reduce and homogenise the size of the liposomal vesicles. Liposomes can be prepared using different techniques. The Lipid film hydration is one of the most implemented liposome formation methods (called: Bangham method), which involves dissolving formulation lipids in an organic solvent, evaporating the organic solvent from mixed lipids, and rehydrating the thin lipid film within an aqueous phase^{6,7,8}. The rehydration of dried film lipids gives rise to multilamellar vesicles (MLVs). Unilamellar vesicles (ULVs) are prepared by subsequent sonication or extrusion. The extrusion through membrane filters gives rise to liposomes with a defined size⁹. Other conventional methods not covered by the present work include detergent removal, ethanol injection, reverse phase evaporation and ether injection¹⁰. An alternative method to produce liposomes with defined size and size distribution is microfluidics^{11,12}. Here, two phases are mixed within intersected microchannels. For example, a water soluble organic phase such as isopropyl alcohol (IPA) is combined with an aqueous phase under laminar flow conditions. Tuning of liposome sizes and distributions can be achieved by adjusting fluid flow rates (volumetric flow rate; VFR), flow rate ratios (FRR), concentrations and compositions of the used lipids¹³.

We were interested in directly comparing the methods of extrusion and microfluidic manufacturing. Therefore, we investigated the variation in the physicochemical properties of fixed lipid formulations manufactured by two different methods: a conventional technique comprising extrusion after drying and then rehydration of a lipids film, and a continuous-flow microfluidic approach. Model liposomes were comprised of dioleoyl phosphatidylcholine (DOPC), phosphatidylserine (PtdSer) and cholesterol. Resulting liposomal formulations were evaluated with respect to size, polydispersity, and ζ -potential¹⁴⁻¹⁹.

Materials and Methods

Materials

DOPC and L- α -PtdSer (dissolved in chloroform, concentration of 25 mg/mL), were purchased from Avanti Polar Lipids (Alabaster, AL, USA). Cholesterol (BioReagent quality) was obtained from Sigma-Aldrich (St. Louis, MO, USA). Dialysis membranes (3500 Da MW cut-off, diameter: 11.5 mm) were purchased from Spectrum Laboratories (Rancho Dominguez, CA, USA). Nuclepore polycarbonate membranes (pore size 0.1 μ m) were obtained from Thermo Fisher Scientific (Waltham, MA, USA). All other chemicals were of analytical quality.

Liposome-microfluidic manufacturing method

To prepare liposomes by the microfluidic method, a NanoAssemblr[®] benchtop instrument was used (Precision NanoSystems, Inc., Vancouver, Canada). Liposomes were prepared at a 1:3 ratio of organic:aqueous solvent with a fixed flow rate of 10 mL/min. Methanol was used as the organic solvent. HEPES buffer (150 mM NaCl, 2 mM CaCl₂, 10 mM HEPES) was used as the aqueous solvent. HEPES was sterile-filtered using 0.22 μ m membranes prior to use in all experiments. Lipids were mixed with methanol followed by sonication at 70°C in a water bath until the lipids were completely dissolved. Liposomes were dialyzed against HEPES buffer to remove organic solvent.

Liposome mini-extrusion manufacturing method

Lipids were dissolved at 1 mg/mL in chloroform. Chloroform was removed using a rotary evaporator using a 5-mL round-bottom flask. The dried lipid film was rehydrated in 3 mL HEPES buffer under stirring. Multilamellar liposomes were extruded using a hand-held extruder (Avanti Polar Lipids). All samples were extruded 13 times through a 100-nm polycarbonate filter membrane positioned between two polyester drain discs.

Physical and chemical characterization of the liposomes by DLS

Size and ζ -potential of the liposomes were measured by dynamic light scattering (DLS; Zetasizer Nano Series, Malvern Instruments, Malvern, UK). The ζ -potential was determined after diluting the samples 1:20 with a 5% glucose solution to reduce the electrode current.

Results and Discussion

Different mol% ratios of cholesterol, DOPC, and PtdSer (Table 1) were prepared using the two liposome preparation methods. Cholesterol content was either 0 mol% or 20 mol%. Increasing amounts of PtdSer were used covering a range from 0 mol% to 10 mol%. Lipid concentrations were always 1 mg/mL.

Table 1: Lipid composition of liposomes used in the present study.

PtdSer: Phosphatidylserine. DOPC: Dioleoyl phosphatidylcholine.

DOPC:PtdSer (mol% ratio)	DOPC:PtdSer:cholesterol (mol% ratio)
100:0	80:0:20
99:1	79.2:0.8:20
95:5	76:4.2:20
90:10	70:10:20

The parameters used for the microfluidic method were 1:3=organic solvent:aqueous solvent and a flow rate of 10 mL/min²⁰.

Liposomes manufactured by the microfluidic method without cholesterol (100:0DOPC:PtdSer) had a size of 34.8 ± 4.3 nm. After adding 1 mol% of PtdSer to the lipid ratio-manufactured liposome (99:1 DOPC:PtdSer), the size (Z-average) increased to 40.3 ± 1.3 nm, likely because of the cone shape structure of PtdSer²¹. A further increase of PtdSer to 10 mol% led to an increase in size of the vesicles (Figure 1). The polydispersity index (PDI) of 100:0 DOPC:PtdSer liposomes was 0.22 ± 0.01 , which was the lowest among the four lipid ratios. The PDI did increase as PtdSer was added, reaching a maximum of 0.37 for all produced liposomes (Figure 2). A PDI > 0.2 can be considered to be representative of a non-uniform size distribution. We conclude that the microfluidics methods yields under standard conditions liposomes with a remarkably small size of down to almost 30 nm at the expense of a non-uniform and variable size distribution (PDI > 0.2). The size increases by a factor of two if increasing amounts of PtdSer are added.

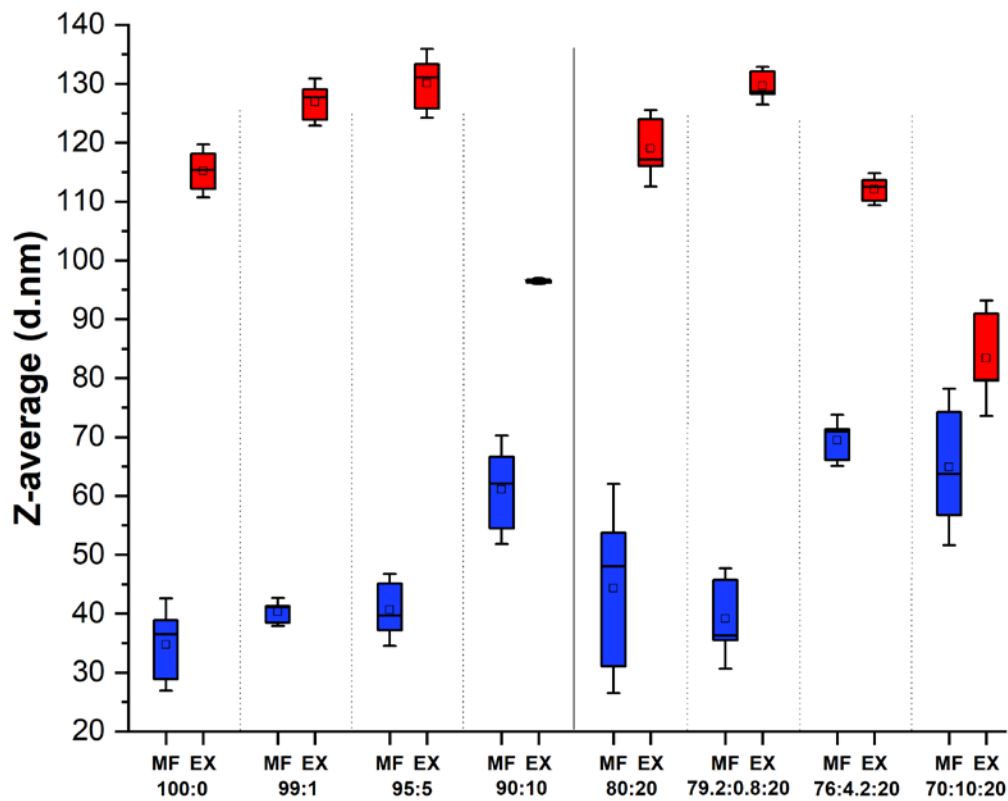


Figure 1: Size of liposomes composed of different lipid mixtures manufactured by microfluidic and extrusion methods. Size (Z-average) of liposomes prepared by a microfluidics (MF, blue bars) or extrusion (EX, red bars) protocol are shown. Indicated lipid ratios: 100:0, 99:1, 95:5, and 90:10 for DOPC:PtdSer; 80:0:20, 79.2:0.8:20, 76:4.2:20, and 70:10:20 for DOPC:PtdSer:cholesterol. Box-plot analysis (median, interquartile range and minima/maxima), $n=3$ independent sets of experiments.

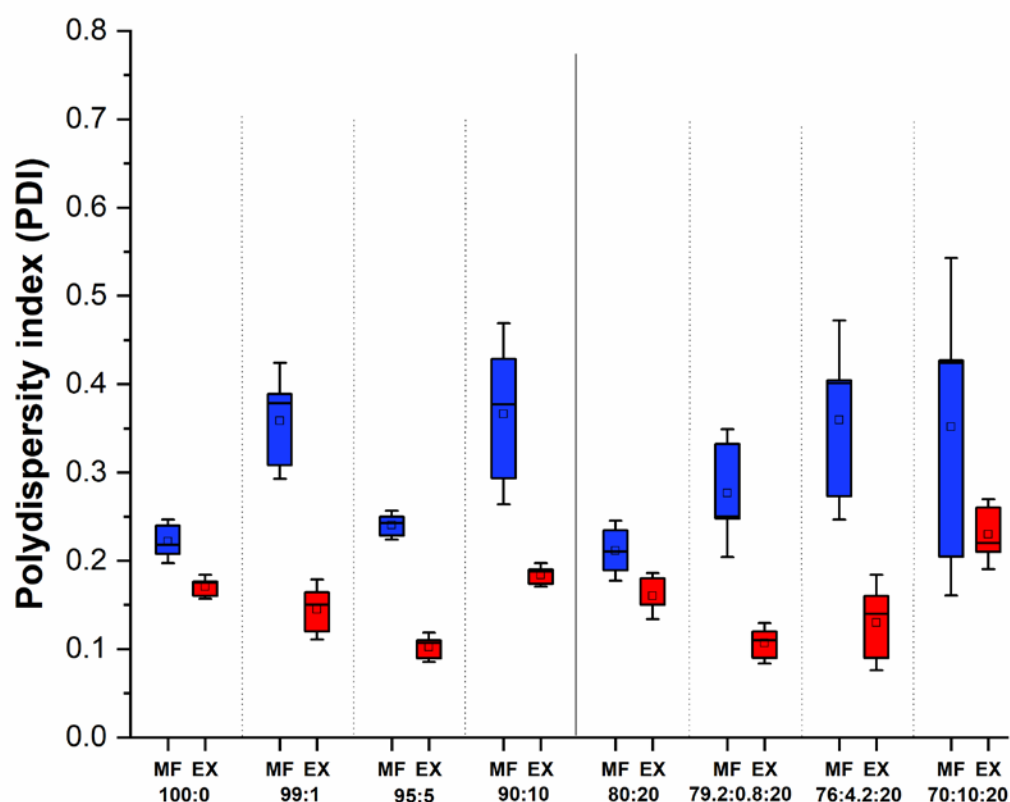


Figure 2: Polydispersity index of liposomes composed of different lipid mixtures manufactured by microfluidic and extrusion methods. Polydispersity index (PDI) of liposomes prepared by a microfluidics (MF, blue bars) or extrusion (EX, red bars) protocol are shown. Indicated lipid ratios: 100:0, 99:1, 95:5, and 90:10 for DOPC:PtdSer; 80:0:20, 79.2:0.8:20, 76:4.2:20, and 70:10:20 for DOPC:PtdSer:cholesterol. Box-plot analysis (median, interquartile range and minima/maxima), $n=3$ independent sets of experiments.

Liposomes prepared by the extrusion method showed an initial gradual increases in size with increasing PtdSer mol% concentrations. In contrast to microfluidics prepared liposomes, the size of these vesicles did decrease at the highest PtdSer concentration (Figure 1). The same phenomenon was observed for liposomes being devoid of cholesterol or containing cholesterol. The PDI of all liposomes was ≤ 0.2 (Figure 2). We conclude that liposomes prepared under standard conditions by

extrusion have a bigger size as compared to their microfluidics counterparts, which have a four-fold smaller size. In contrast to the microfluidics protocol, the extruded liposomes have a monodisperse size distribution.

The 100:0 DOPC:PtdSer liposomes manufactured by the microfluidic and extrusion methods showed a positive ζ -potential of 2.8 ± 0.4 and 0.8 ± 0.9 mV, respectively. Minor differences in charge were observed between the two manufacturing methods, possibly because of differences in their surface areas, as both methods produced liposomes with significantly different sizes. In presence of increasing amounts of PtdSer, the ζ -potentials of liposomes manufactured by both methods decreased linearly. At the highest PtdSer concentration of 10 mol%, values were -30.9 ± 3.0 mV for microfluidics preparations and -27.7 ± 2.5 mV for extruded liposomes, respectively (values are means \pm SD, n=3). This result is expected due to the negative charge of PtdSer under physiological conditions. The impact of cholesterol on the ζ -potential was negligible (Figures 3 and 4).

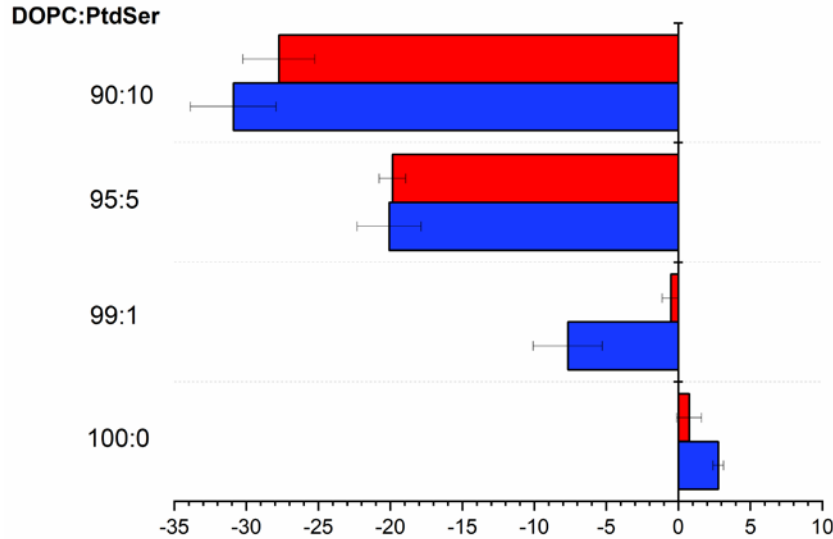


Figure 3: ζ -potential of liposomes with different lipids ratios prepared without cholesterol. Blue bars: microfluidic manufactured liposomes, Red bars: extrusion manufactured liposomes. Lipids ratios: 100:0, 99:1, 95:5, and 90:10 DOPC:PtdSer. Values are means \pm SD, $n=3$.

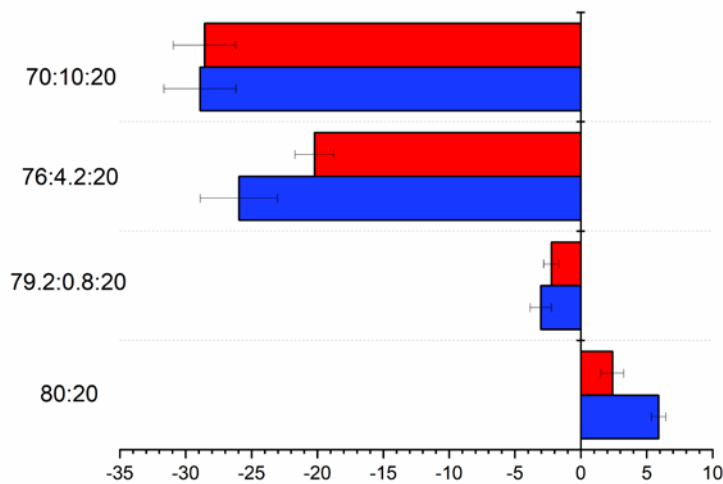


Figure 4: ζ -potential of liposomes with different lipids ratios containing 20 mol% cholesterol. Blue bars: microfluidic manufactured liposomes, Red bars: extrusion manufactured liposomes. Lipid ratios: 80:0:20, 79.2:0.8:20, 76:4.2:20, and 70:10:20 DOPC:PtdSer:cholesterol. Values are means \pm SD, $n=3$.

Conclusion

Our experiments demonstrate that liposomes prepared under standard conditions, using either a microfluidics or an extrusion protocol, have significantly different properties. It is tempting to assume that microfluidics led in our experiments to higher shear stress during the formation of liposomes and thus a smaller size. However, due to geometric constraints of the lipid membrane curvature, such small liposomes seem to be less stable than their bigger counterparts leading to a high PDI. Intermembrane fusion occurs more readily in small vesicles as this process relieves thermodynamically unfavorable packing constraints²². Surprisingly, PtdSer has a destabilizing effect in microfluidics prepared smaller liposomes leading to a bigger size and PDI and the opposite effect in bigger extruded liposomes. This effect could be caused by the 2 mM Ca^{2+} present in the incubation buffer since this bivalent cation has been shown to interact with the headgroups of PtdSer, joining them together, and inducing their translocation to the inner leaflet of the bilayer. This effect is size dependent and more pronounced for smaller²³.

Our findings should be considered when a formulation that has been developed for extrusion is adapted to a microfluidic process. Depending in the used method, not only the size and polydispersity of the liposomes will change but there might be additional effects on long term stability and possibly the asymmetric degree of lipid distribution within the membrane bilayer.

References

- 1 Bulbake, U., Doppalapudi, S., Kommineni, N., et al., 2017. Liposomal formulations in clinical use: an updated review. *Pharmaceutics*, 9 (2), 12.
- 2 Bozzuto, G., Molinari, A., 2015. Liposomes as nanomedical devices. *International journal of nanomedicine*, 10, 975-999.
- 3 Yusaf, R., Nawaz, R., et al., 2014. Structural components of liposomes and characterization tools. *Indo American Journal of Pharmaceutical Research*, 4.
- 4 Birge, R.B., Herrmann, M., et al., 2016. Phosphatidylserine is a global immunosuppressive signal in efferocytosis, infectious disease, and cancer. *Cell Death Differentiation*, 23(6), 962-78.
- 5 Mozafari, M.R., 2005. Liposomes: an overview of manufacturing techniques. *Cellular and Molecular Biology Letters*, 10 (4), 711–719.
- 6 Bangham, A.D., Standish, M.M., Watkins J.C., 1965. Diffusion of univalent ions across the lamellae of swollen phospholipids. *Journal of Molecular Biology*, 13(1), 238-252.
- 7 Bangham A.D., Standish, M.M., Weissmann, G., 1965. The action of steroids and streptolysin S on the permeability of phospholipid structures to cations. *Journal of Molecular Biology*, 13(1), 253-259.
- 8 Maherani, B., Arab-Tehrany, E., Mozafari, M.R., et al., 2011. Liposomes: a review of manufacturing techniques and targeting strategies. *Current Nanoscience*, 7, 436-452.
- 9 Çağdaş, M., Sezer, A.D., Bucak, S., 2014. Liposomes as potential drug carrier systems for drug delivery. In: *Application of Nanotechnology in Drug Delivery*. London: InTech.
- 10 Meure, L.A., Foster, N.R., Dehghani, F., 2008. Conventional and dense gas techniques for the production of liposomes: a review. *AAPS PharmSciTech*, 9 (3), 798.
- 11 Zhigaltsev, I.V., Cullis, P.R., 2012. Bottom-up design and synthesis of limit size lipid nanoparticle systems with aqueous and triglyceride cores using millisecond microfluidic mixing. *Langmuir*, 28 (7), 3633–3640.
- 12 Woodle, M.C., Papahadjopoulos, D., 1989. Liposome preparation and size characterization. *Methods in Enzymology*, 171 (C), 193-217.
- 13 Yu, B., Lee, R.J., Lee, L.J., 2009. Microfluidic methods for production of liposomes. *Methods in Enzymology*, 465, 129-141.
- 14 Hoshyar, N., Gray, S., Han, H., et al., 2016. The effect of nanoparticle size on in vivo pharmacokinetics and cellular interaction. *Nanomedicine (Lond.)*, 11 (6), 673-692.

- 15 Mudalige, T., Qu, H., Van Haute, D., et al., 2019. Characterization of nanomaterials: tools and challenges. *In: Nanomaterials for Food Applications*. New York, NY: Elsevier, 313–353.
- 16 Clayton, K.N., Salameh, J.W., Wereley, S.T., et al., 2016. Physical characterization of nanoparticle size and surface modification using particle scattering diffusometry. *Biomicrofluidics*, 10 (5), 054107.
- 17 Malvern Instruments Ltd., 2014. *What Is The Z Average?* [online]. Available from: <http://www.materials-talks.com>.
- 18 Sharma N., Verma, S. 2017. Current and future prospective of liposomes as drug delivery vehicles for the effective treatment of cancer. *International Journal of Green Pharmacy*, 11 (3), S377.
- 19 Smith, M.C., Crist, R.M., Clogston, J.D., et al., 2017. Zeta potential: a case study of cationic, anionic, and neutral liposomes. *Anal Bioanal Chem*, 409 (24), 5779-5787.
- 20 Kotouček, J., Hubatka, F., Mašek, J., et al., 2020. Preparation of nanoliposomes by microfluidic mixing in herring-bone channel and the role of membrane fluidity in liposomes formation. *Scientific Reports*, 10, 5595.
- 21 Li, J., Wang, X., Zhang, T., et al., 2015. A review on phospholipids and their main applications in drug delivery systems. *Asian Journal of Pharmaceutical Sciences*, (10), 81–98.
- 22 New, R.R.C., 1990. Liposomes, a practical approach. *Oxford University Press*.
- 23 Yue Guo, Hao, 2020. Fabrication of Asymmetric Phosphatidylserine-Containing Lipid Vesicles: A Study on the Effects of Size, Temperature, and Lipid Composition. *Langmuir*, 36, 12684–12691.