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# Cross-flow filtration—an improved detergent removal technique for the preparation of liposomes

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**Abstract**

Using a cross-flow (tangential) filtration device, a lipid-detergent mixed micellar solution is filtered under pressure through a suitable membrane with a molecular weight exclusion limit of 10–50 kDa. In contrast to commonly used dead-end ultrafiltration filters, the solution flows tangential to the membrane surface. A wave-like flow of the solution over the membrane is effected due to the shape of the membrane, preventing clogging during filtration. Detergent monomers pass through the membrane and are collected as waste filtrate, whereas the mixed micelle solution is continuously pumped as retentate through the device until liposome formation is completed. Buffer solution is introduced into the system at the same rate as the filtrate is removed therefrom to keep the retentate volume constant. Variations of the following parameters were studied in detail: (1) membrane cutoff; (2) filtration area; (3) flow rate; (4) starting lipid/detergent ratio; (5) choice of detergent; and (6) cholesterol content. We determined the kinetics of detergent removal and compared filtration time, amount of filtrate, vesicle size and stability of the resulting liposome dispersions. Cross-flow filtration under optimum conditions results in liposome formation within minutes and in principle makes unlimited upscaling possible. 

*Keywords:* Liposome preparation; Mixed micelles; Detergent removal; Cross-flow filtration

## 1. Introduction

Detergent-mediated liposome production is based on the solubilization of lipid with the aid of a suitable detergent resulting in the formation of mixed micelles (Helenius and Simons, 1975; Lichtenberg et al., 1983). Detergents bound to the edges of the disk-shaped mixed micelles (Mazer et al., 1976) are in equilibrium with free monomers in solution (Schubert and Schmidt, 1988). While lowering the concentration of free monomers, detergent is removed mainly from the edges of the mixed micelles. This leads to a reduction of the

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length of the edges and meanwhile increases lipid packing, which enhances lateral fusion and growth of the micelles. The edge tension increases and induces a curvature in the disks. The extent of membrane curvature depends on the type of detergent and, therefore, determines the liposome diameter (Fromherz et al., 1986). Finally the curved bilayers close to form unilamellar vesicles. The membranes still contain initially up to 25 mol.% of detergent. These membrane-bound detergents induce severe effects in the bilayer, i.e. strong increase in membrane fluidity (decrease in structural order parameter) and number of membrane defects, which in turn can lead to uncontrollable vesicle fusion, resulting in inhomogeneous vesicle size and increased lamellarity (Schubert et al., 1986). Fast removal of membrane-bound detergent is, therefore, a crucial step in detergent removal techniques. The accelerated lowering of detergent monomer concentration can be achieved by means of dilution (Schurtenberger et al., 1984) or gel chromatography (Brunner et al., 1976), fast and controlled dialysis through thin cellulose membranes (Milsmann et al., 1978) or dialysis through hollow fibers (Rhoden and Goldin, 1979). The use of dialysis bags only leads to slow removal of detergent and may result in reduced liposome quality.

The detergent removal techniques mentioned above are time consuming and only suitable for a small scale production. Moreover, when using relatively high lipid concentrations (more than 20 mM) to enhance the yield of liposomes and the trapping efficiency of hydrophilic drugs, the use of detergents leads to an increase in the number of lamellae.

In general, however, detergent removal is a technique which permits the production of homogeneous unilamellar liposomes with tailored size by the choice of a suitable detergent (Schwendener et al., 1981; Zumbuehl and Weder, 1981). A procedure is, therefore, needed which combines the advantages of the known detergent removal techniques with an accelerated and efficient removal of detergent, thus strongly reducing preparation time and undesired liposome structures at higher lipid concentrations. In addition, the procedure should be economic and preferable even to be carried out continuously and under sterile conditions.

The present work describes an improved technique of detergent removal to overcome the limitations of the known removal techniques.

## 2. Materials and methods

### 2.1. Material

Soya-lecithin (S-100) was a generous gift from Lipoid (Ludwigshafen, Germany). Sodium cholate, cholesterol and MOPS were purchased from Sigma (Deissenhofen, Germany). *N*-octyl $\beta$-D-glucopyranoside was obtained from Calbiochem (Bad Soden, Germany). All other chemicals were of analytical grade.

The tangential filtration unit consisted of a Minisette stainless steel cassette hardware, a Minisette cassette Omega screen channel 10 kDa (filtration area 0.75 $ft^2$, ca. 700 $cm^2$) or alternatively a Minisette single Omega membrane 10 kDa (filtration area 0.15 $ft^2$, ca. 140 $cm^2$) from Pall Filtron (Dreieich, Germany). The material of the membranes was modified polyether-sulfone with low protein binding. Pump (7524-05) and tubings (16′) were used from Masterflex (USA).

### 2.2. Mixed micelle solutions

All components to form the mixed micelles were dissolved in organic solvent (methanol/chloroform) in a round bottom flask. The final concentration per batch (100 ml) was 10 mM lipid, either exclusively soya-lecithin S-100 or a mixture of S-100 and cholesterol (33 mol.%). The average lipid/detergent ratio was 1:1.6 (mol/mol) for sodium cholate (for some experiments this ratio was varied to 1:1.4 and 1:8). For the use of *n*-octyl $\beta$-D-glucopyranoside the lipid/detergent ratio was 1:5 (mol/mol). The organic solvent was subsequently removed by rotary evaporation and the lipid film was dried under vacuum for another 60 min. MOPS-buffer (10 mM with 150 mM NaCl, pH 7.3) was added up to 100 g and the film was resuspended resulting in a clear mixed micelle solution.

### 2.3. Tangential filtration process

The tangential filtration unit consisted of a starting reservoir, a pump, the filtration device (membrane) and tubings with integrated rotary slide valve and a manometer to follow the retentate pressure $P_R$ (Fig. 1). By increasing the retentate pressure $P_R$, the pressure on the membrane increases which leads to faster removal of detergent.

The starting reservoir contained the mixed micelle solution which was subjected to tangential filtration. The filtration unit was equipped with a single membrane or membrane cassettes with a cutoff of either 10 or 50 kDa. The flow rate was adjusted to 75 or 250 ml/min. The filtration was carried out at a resulting $P_R$ of 0.4 bar. The volume of filtered out detergent-containing filtrate was substituted by an equal volume of MOPS-buffer (volumetric control). All experiments were performed at least in triplicate to study reproducibility.

### 2.4. Residual cholate concentration

The cholate concentration was analyzed before the filtration and at a certain time points in the retentate by HPLC (Peschka, 1994) with an equipment from Kontron (Neufarn, Germany) using a LiChrospher 60 RP-select B column from Merck (Darmstadt, Germany), $150 \times 4.0$ mm, 5 $\mu$m. The eluent consisted of 1 g octansulfonic acid (sodium salt), 5 g perchloric acid, 750 g HPLC-water and 350 g acetonitrile. The detection wave length was 220 nm at a flow rate of 1 ml/min and an injection volume of 25 $\mu$l. The retention time of the cholate-peak was at 5.1 min. The samples were injected without further treatment.

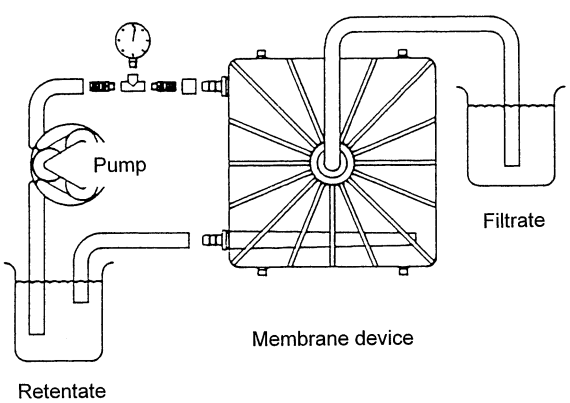


Fig. 1. Schematic drawing of the experimental setup. A cross-flow filtration unit consists of a starting reservoir (retentate), a pump, the filtration device with the membrane and a manometer to follow the retentate pressure $P_R$.

### 2.5. Vesicle size

The size of the liposomes was determined by photon correlation spectroscopy (Zetamaster®, Malvern, Herrsching, Germany). Vesicle sizes given in this paper reveal the $z$-average of three measurements and the S.D.

### 2.6. Lipid concentration

The lipid concentration of the final liposome dispersion was determined by a phosphorous assay (Bartlett, 1959).

## 3. Results and discussion

### 3.1. Membrane cutoff

Membranes with a cutoff of 10, 30, 50 and 100 kDa were used. It showed that when using a membrane cutoff of 100 kDa, lipid containing associates were found in the filtrate, which indicates that the membrane pores were too large to hold back the mixed micelles in the system. In all other cases there was no loss of lipid throughout the filtration process.

The kinetics of detergent removal using single membranes with a filtration area of 0.15 ft$^2$ (140 cm$^2$) and a cutoff of either 10 or 50 kDa are displayed in Fig. 2. There are no significant differences concerning filtration time, vesicle size and filtrate. It took either 240 min (with the 10 kDa membrane) or 270 min (50 kDa membrane) until the molar cholate/lipid ratio was less than 0.3 (Table 1). It was shown earlier (Schubert and Schmidt, 1988) that at a molar ratio of bound bile salt to total membrane lipid of 0.3, no micellar structures are existing any longer. Therefore, at a

Table 1
Variation of cutoff, filtration area and flow rate

| Membrane cut-off (kDa) | Filtration area ($ft^2$) | Flow rate (ml/min) | Filtration time until cholate/lipid $<0.3$ (molar ratio) (min) | End of filtration | | Filtrate (ml) | Vesicle size (nm) | |
|---|---|---|---|---|---|---|---|---|
| | | | | (min) | Cholate/lipid (mol/mol) | | After production | After 10 months |
| 10 | 0.15 | 250 | 240 | 360 | 0.09 | 530 | $47.4 \pm 7.7$ | $49.5 \pm 13.3$ |
| 50 | 0.15 | 250 | 270 | 360 | 0.19 | 450 | $55.0 \pm 11.6$ | $55.2 \pm 11.3$ |
| 10 | 0.75 | 250 | 12 | 60 | 0.01 | 300 | $45.7 \pm 8.2$ | $44.7 \pm 10.9$ |
| 10 | 0.75 | 75 | 27 | 60 | 0.08 | 400 | $47.6 \pm 9.4$ | $42.0 \pm 10.5$ |

Lipid, 10 mM S-100; detergent, sodium cholate; lipid/detergent, 1:1.6 (mol/mol); buffer, MOPS pH 7.3; and $P_R$, 0.4 bar.
Data in this table reveal the end of filtration and filtration time needed for a residual cholate/lipid ratio (mol/mol) less than 0.3. Below this ratio liposome formation is completed. Nevertheless the process of detergent removal was continued further on to avoid structural changes in the liposomal membrane during storage.

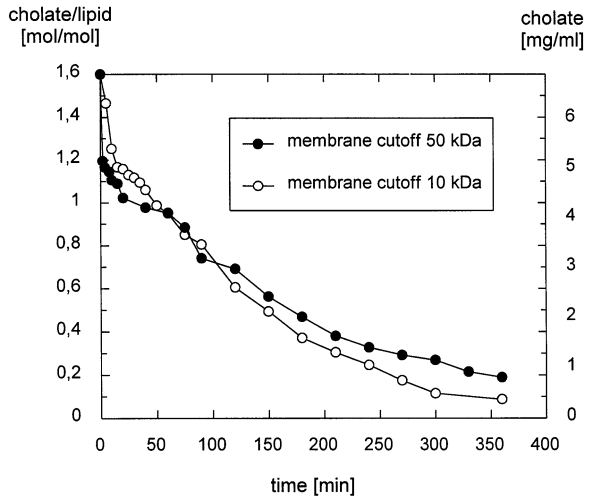


Fig. 2. Kinetics of detergent removal using different membrane cutoffs. Lipid, 10 mM S-100; detergent, sodium cholate; lipid/detergent, 1:1.6 (mol/mol); buffer, MOPS pH 7.3; filtration area of membrane, 0.15 ft$^2$ (140 cm$^2$); flow-rate, 250 ml/min; $P_R$, 0.4 bar.

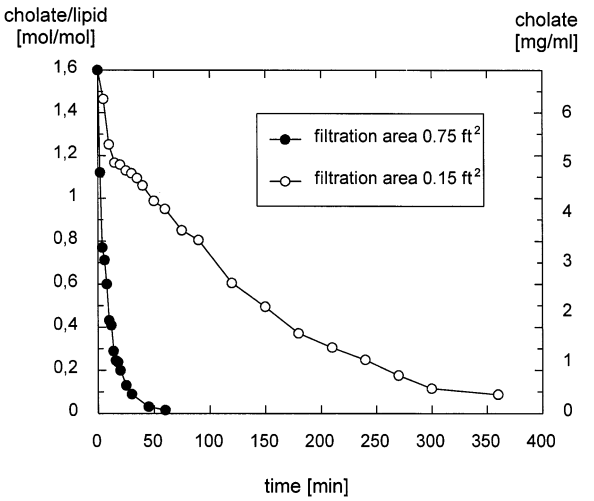


Fig. 3. Kinetics of detergent removal using different filtration areas. Lipid, 10 mM S-100; detergent, sodium cholate; lipid/detergent, 1:1.6 (mol/mol); buffer, MOPS pH 7.3; membrane cutoff, 10 kDa; flow-rate, 250 ml/min; $P_R$, 0.4 bar.

molar ratio of total (bound and free) cholate to lipid less than 0.3, liposome formation is certainly completed. At the end of the filtration after 360 min there was less waste filtrate when using a 50 kDa membrane. Vesicle sizes and S.D. were similiar. There was no increase in vesicle size in both preparations after 10 months storage.

Obviously different cutoffs of the filtration membrane do not essentially influence the studied parameters.

### 3.2. Filtration area

Either a single membrane with a filtration area of 0.15 ft$^2$ (140 cm$^2$) or a membrane cassette with a filtration area of 0.75 ft$^2$ (700 cm$^2$) was used. Both membranes had a cutoff of 10 kDa. As shown in Fig. 3, the kinetics of detergent removal are highly influenced by the filtration area. The increase in filtration area by a factor of 5 reduces filtration time by a factor of 20 (Table 1). Also the volume of waste filtrate was reduced. Vesicle sizes were almost identical with a stability over at least 10 months.

It showed that scaling up the filtration area dramatically reduces time of liposome formation while not influencing the quality of the liposomes.

### 3.3. Flow rate

The flow rate was either adjusted to 75 or 250 ml/min. Filtration time until liposome formation was reduced upon increasing the flow rate (Fig. 4) while not influencing vesicle size and stability (Table 1).

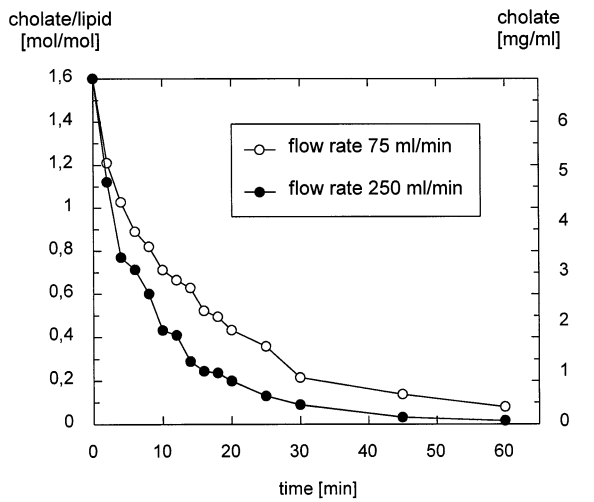


Fig. 4. Kinetics of detergent removal using different flow rates. Lipid, 10 mM S-100; detergent, sodium cholate; lipid/detergent, 1:1.6 (mol/mol); buffer, MOPS pH 7.3; membrane cutoff, 10 kDa; filtration area, 0.75 ft$^2$ (700 cm$^2$); $P_R$, 0.4 bar.

Table 2
Influence of lipid/cholate ratio and cholesterol on vesicle size

| Lipid | Lipid/cholate (mol/mol) | Vesicle size (nm) | |
|---|---|---|---|
| | | After production | After 10 months |
| S-100 | 1:1.4 | $56.7 \pm 9.8$ | $61.5 \pm 13.3$ |
| S-100 | 1:1.6 | $44.8 \pm 8.2$ | $44.7 \pm 10.9$ |
| S-100/cholesterol 2:1 (mol/mol) | 1:1.6 | $44.3 \pm 8.9$ | $40.6 \pm 9.9$ |
| S-100 | 1:8.5 | $35.3 \pm 8.1$ | $41.7 \pm 11.8$ |

Lipid, 10 mM; membrane cutoff, 10 kDa; filtration area, 0.75 $ft^2$ (700 $cm^2$); detergent, sodium cholate; flow rate, 250 ml/min; buffer, MOPS pH 7.3; and $P_R$, 0.4 bar.

### 3.4. Lipid/detergent ratio

A lipid/detergent ratio of at least 1:1.2 is required to solubilize lipids when sodium cholate is used as detergent (Schubert et al., 1986). In our experiments we used different ratios above this amount to study the influence of the starting lipid/detergent concentration in the mixed micelle solution (Table 2). A slight decrease in vesicle size was observed upon increasing the initial cholate concentration. It has to be noted that a high starting concentration of detergent increases production time and waste filtrate (data not shown).

### 3.5. Choice of detergent

As shown in Table 3, the size of liposomes can be tailored by the choice of detergents and mixtures thereof. Vesicles prepared with sodium cholate are significantly smaller than with octylglucoside, which has been reported also with detergent dialysis (Schwendener et al., 1981; Zumbuehl and Weder, 1981). A 1:3 mixture of sodium cholate and octylglucoside results in small vesicles comparable to vesicles prepared with sodium cholate alone. This leads to the conclusion that octylglucoside is removed faster during filtration than cholate and that the vesicle curvature is mainly controlled by the remaining cholate.

### 3.6. Influence of cholesterol

Surprisingly there was no influence at all on filtration time, vesicle size and waste filtrate comparing soya-lecithin and soya-lecithin compositions with 33 mol.% cholesterol (Table 2). This effect may be explained by the fact that detergent monomers are probably removed differently comparing slow and fast removal. When removing the detergent monomers slowly from the mixed micelles, detergent molecules are released from the edges as well as from the flat surface of the disks. The presence of cholesterol then strongly influences the rigidity of the membrane which results in a decreased curvature (increased diameter) of the liposomes. By the process of tangential filtration the removal of detergent is very fast and detergent molecules are removed mainly from the edges of the disks. Residual detergents on the flat surface weaken the stiffening effect of cholesterol, resulting in the same vesicle sizes of the final liposome dispersions.

## 4. Conclusion

The process of cross-flow filtration leads to liposomes of defined size, homogeneity and high stability. Large quantities of liposomes can be produced in significant shorter time than with any other method used for detergent removal. Large scale liposome production is possible by the increase of filtration area using larger cassette holders and connecting several membrane cassettes in series. Also sterile products can be obtained by the presented method when starting with sterile filtered mixed micelles and autoclaved devices. The production can be carried out continuously and the resulting liposome dispersion can be concentrated at the end of the process by no longer

Table 3
Influence of detergent on vesicle size

| Detergent | Lipid/detergent (molar ratio) | Vesicle size (nm) | |
|---|---|---|---|
| | | After production | After 10 months |
| Sodium cholate | 1:1.6 | $55.3 \pm 11.8$ | $55.4 \pm 11.9$ |
| Sodium cholate/*n*-octyl $\beta$-D-glucopyranoside | 1:1:3 | $60.3 \pm 10.8$ | $52.5 \pm 13.1$ |
| *n*-Octyl $\beta$-D-glucopyranoside | 1:5 | $144.9 \pm 33.3$ | $150.6 \pm 30.6$ |

Lipid, 10 mM S-100; membrane cutoff, 50 kDa; filtration area, 0.15 $ft^2$ (140 $cm^2$); flow rate, 250 ml/min; buffer, MOPS pH 7.3; and $P_R$, 0.4 bar.

substituting the waste filtrate in the retentate. The waste filtrate can be recycled to minimize costs of production.

The present work demonstrates with some preliminary data that liposome production by cross-flow filtration improves the known detergent removal techniques. More parameters, such as maximum lipid concentration, temperature, trapping efficiency for hydrohilic drugs have to be studied in detail to gain more information about this method.

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