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LARGE-SCALE PRODUCTION OF LIPOSOMES OF DEFINED SIZE BY A NEW CONTINUOUS HIGH PRESSURE EXTRUSION DEVICE

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ABSTRACT

A new continuous high pressure extrusion apparatus for the generation of liposomes was designed and tested in the present study. The extruder, which basically consists of an open supply vessel and a high pressure filter holder mounted on a gas-driven pump exhibited superior abilities compared to currently available, discontinuous extrusion devices. Due to continuous flows up to 500 ml/min (filter diameter 47 mm), large batches on a liter scale could be extruded in one step. The maximum pressure of 10.5 MPa employed here, enabled the rapid passage of liposomal preparations with various lipid compositions at lipid concentrations as high as 400 mg/g through polycarbonate membranes without any clogging of the system. Employing final pore sizes between 5.0 and 0.03 μm, the mean vesicle diameter of liposomal preparations could be varied from 400 to 60 nm. Surprisingly high encapsulation efficiencies of two water-soluble contrast agents, iopromide and Gd-DTPA, which we used as model substances, were obtained for continuously extruded liposomes. Values of

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over 50 % for iopromide and more than 60 % for Gd-DTPA , which were achieved in combination with a freeze-thaw protocol, to our knowledge exceed all results reported so far for passive entrapment of water-soluble contrast agents. The generation of 6 batches of iopromide-carrying liposomes under identical conditions revealed good reproducibility of the new method. Physico-chemical characterization of void as well as contrast-carrying liposomes after storage at 2-8° C for 6 months showed satisfactory long-term stability of continuously extruded liposomes.

The new structural design of the high pressure extrusion device permits, for the first time, reproducible, continuous, large-scale production of stable liposomes of defined size. Scaling up the widely used extrusion method to an industrial level seems feasible now.

INTRODUCTION

Liposomes, small lipid vesicles composed of one or several concentric bilayers, similar to cell membranes, are widely used as carriers for therapeutics and diagnostics. Bilayers form as soon as certain amphiphatic lipids, e.g. phosphatidylcholine, are dispersed in aqueous solutions. During the formation of tightly closed spheres, water-soluble substances can be entrapped within the aqueous core of the vesicle or in the space between two or more bilayers. Different groups of liposomes are distinguished by their size and lamellarity. Small unilamellar vesicles (SUV) consist of one bilayer and measure around 100 nm or less, whereas large unilamellar vesicles (LUV) can have diameters of several hundred nm. Oligolamellar and multilamellar vesicles (OLV and MLV) consist of several or many bilayers and can reach diameters of more than 1 μm.

Various methods for the generation of liposomes have already been developed. Besides high pressure homogenization methods like the one-step method [1], which permits the rapid generation of small liposomes with narrow size distribution, lately, the so-called extrusion method has drawn wide attention because it produces vesicles with a whole range of different defined sizes [2, 3]. When using the term extrusion, we refer to a technique, reported for the first time in 1979 by Olson et al. [2], which

employs high pressures in order to force liposomes through membranes (e.g. of polycarbonate) with defined straight pores. During the filter passage, liposomes larger than the pore size of the membrane are destroyed and smaller vesicles arise from the bilayer fragments. Beside its abiltity to vary the liposome size, extrusion has additional advantages: (1) It yields homogeneous size distributions when small final pore sizes are employed [2, 4]. (2) Neither solvents nor detergents have to be removed from the final product [5]. (3) Due to the filtration process, the absence of particulate impurities is guaranteed [6]. (4) A variety of different lipids, sometimes at high concentrations, can be used [5].

Even though conventional extrusion methods are already widely used, two main obstacles to successful upscaling to an industrial level remain: The limited volume capacity as well as relatively low maximum pressures of most currently available extruders. These discontinuous devices, which work with pressures up to 5.5 MPa usually only allow extrusion of 50 ml or less [4, 7, 8]. To our knowledge, just one device exists which can withstand higher pressures (up to 10.5 MPa), but which is also restricted to a maximum volume, in this case of 400 ml. [9]. All these extruders consist of a pressure filtration chamber with a certain volume that determines the upper limit of batch sizes. Capacities and maximum pressures reported so far for discontinuous systems appeared insufficient for our purpose, which includes the use of large volumes of liposome suspensions, sometimes at high lipid concentrations, and the employment of lipids with high phase transition temperatures. We have, therefore, designed and tested a new high pressure extrusion apparatus which allows the continuous extrusion of large batches at pressures up to 10.5 MPa.

In this work, we attempted to evaluate the new system, producing various liposomal preparations. The impact of process parameters like lipid composition and concentration as well as solute concentration on vesicle size distribution, trapping efficiencies and stability of continuously extruded liposomes was investigated. In some cases, we used the new method in combination with a freeze-thaw technique, which was already described for the improvement of solute encapsulation in 1981 [10]. Our results indicate that the fast and reproducible generation of large batches

of stable liposomes with defined size and high encapsulation efficiencies has now become possible.

MATERIALS AND METHODS

Lipids

Soy phosphatidylcholine (Lipoid S100®, SPC), egg phosphatidylcholine (Lipoid E 100®, EPC), hydrated soy phosphatidylcholine (Lipoid SPC 3®, hydr.SPC), soy phosphatidylethanolamine (Lipoid SPE®, SPE), egg phosphatidylserine (Lipoid EPS®, EPS), soy phosphatidylglycerol (Lipoid SPG®, SPG) and soy phosphatidic acid (Lipoid SPA®, SPA) were obtained from Lipoid KG, Ludwigshafen, Germany. Distearoyl phosphatidylcholine (DSPC) was from Sygena Ltd., Liestal, Switzerland. Dicetylphosphate (DCP) was from Sigma Chemical Co., St. Louis, MO, USA. Cholesterol (Chol) was from Solvay Duphar B.V., Veenendal, The Netherlands. Stearic acid (SS) was obtained from Fluka Chemie, Buchs, Switzerland. All lipids were used without further purification. EPS, SPG, SPA and DCP are negatively charged, all the other substances display no net charge.

Contrast Agents

Ultravist® 370, a ready-to-use preparation containing the non-ionic, water-soluble x-ray contrast agent iopromide (mol. weight 791.14 g) in tromethamine buffer as well as Magnevist®, containing the ionic, water-soluble paramagnetic gadolinium diethylenetriamine pentaacetic acid (Gd-DTPA) as its dimeglumine salt (mol. weight 938.01 g) were obtained from Schering AG, Berlin, Germany. Contrast agent solutions were diluted to the desired concentration with either 20 mM tromethamine buffer, pH 7.5, (Ultravist®) or bidistilled water (Magnevist®).

The Continuous High Pressure Extrusion Apparatus

The continuous high pressure extrusion apparatus (Maximator® Model HPE 10.0 - 250, Schmidt, Kranz & Co, Zorge, Germany) consists mainly of an open supply vessel for the liposomal preparation and a high

pressure filter holder, which is mounted on a gas-driven pump (FIGURE 1). The principle of liposome extrusion with this new device is to force large multilamellar vesicles, which were filled into the supply vessel, continuously through polycarbonate membranes that are embedded in the high pressure filter holder. The apparatus allows control of the incoming as well as the resulting pressure in the system (extrusion pressure). Due to the filter holder construction the maximum extrusion pressure which we could employ with this prototype apparatus was 10.5 MPa. Since the extruder works with continuous flows (up to 500 ml/min) large volumes can be passed through the polycarbonate membranes in one step. Two options exist for extruded vesicles. They are either recirculated into the supply vessel, so that automatic, continuous extrusion for a certain time period is achieved, or they are collected in a seperate vessel and extruded again as a whole batch, in order to determine a desired number of passages. There is no maximum batch size for the apparatus itself, the filtration flow is, however, limited by the diameter of the employed polycarbonate membranes (47 mm). Thus, the use of filters with larger surfaces might be necessary for further scaling up of the process.

Preparation of Liposomes

MLV for extrusion were usually generated by the film method. Briefly, lipids were dissolved in ethanol (96%, reagent grade, from E. Merck, Darmstadt, Germany) at elevated temperatures (50-60° C). After removal of the solvent on a rotary evaporator (90 min at 15-30 mbar and 50° C, Rotavapor-R, Büchi, Flawil, Switzerland), a thin film on the walls of a round bottom flask was obtained. Buffer or contrast agent solutions in water were used either at room temperature or after heating above the phase transition temperature of lipids to suspend the lipid film. Extrusion was carried out at these temperatures using polycarbonate membranes (47 mm, Nuclepore GmbH, Tübingen, Germany) with pore diameters of 5.0, 1.0, 0.4, 0.2, 0.1, 0.05 and 0.03 µm. Liposomes were passed through two stacked membranes, usually 5 times for each pore size. All filters were employed in a sequence of decreasing pore sizes, as mentioned above. For the generation of different mean vesicle diameters, however,

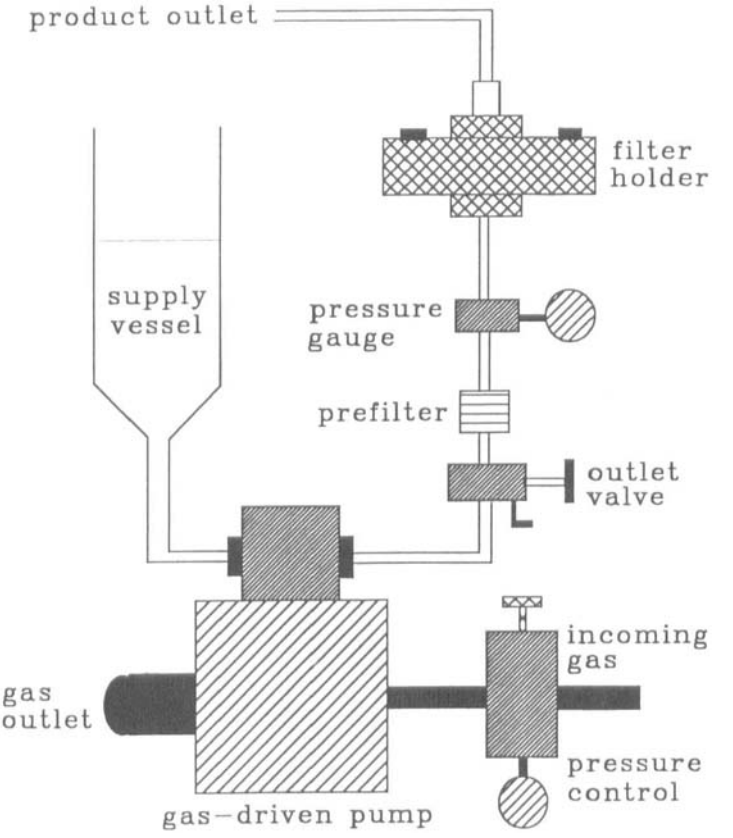

FIGURE 1

Schematic diagram of the continuous high pressure extrusion apparatus.

we simply varied the pore sizes for the final extrusion step. Afterwards, liposomes were filtered through microbe retentive cellulose acetate filters (0.22 μm, Satorius, Göttingen, Germany) and filled into sterile glass vials under aseptic conditions. During the freeze-thaw procedure, liposomes were frozen in glass vials at -70° C in Methanol/CO_2 and thawed at 70° C in a water bath. The protocol was carried out three times after passage through 0.4 μm pore size and before continuing further extrusion.

Determination of Liposome Size

Liposome size distribution was determined by photon correlation spectroscopy (PCS) using a Submicron Particle-Sizer Autodilute®, Model

370, Nicomp Instr. Corp., Santa Barbara, CA, USA. The volume-weighted gaussian analysis modus was employed for the calculation of mean vesicle diameters and variation coefficients (CV), after 10 minutes of measurement at an angle of 90° and a temperature of 25° C.

Encapsulation Efficiency

Encapsulation efficiency (amount of encapsulated contrast agent in the preparation given as percent of total recovered), also referred to as entrapment value or trapping efficiency, was determined by equilibrium dialysis, using a Dianorm system, Dianorm, Munich, Germany. Iopromide concentration, total and unencapsulated, was measured by UV photometry at 242.6 nm with a UV/VIS Spectrometer Lambda 2, Perkin Elmer, Überlingen, Germany. Gd concentration, total and unencapsulated, was determined by inductively coupled plasma atomic emission spectrometry (ICP-AES), using an ICP-AES Modell Plasma 1000, Perkin Elmer, Überlingen, Germany. Prior to determination of the total contrast agent concentration, the liposomes had to be destroyed. For iopromide analysis this was done by dilution of small aliquots of the liposomal preparation with methanol (spectroscopic grade, Schering AG, Berlin, Germany). Destruction of Gd-DTPA liposomes was carried out by application of high pressures and elevated temperatures in 32% HNO_3 (E. Merck, Darmstadt, Germany) using high pressure vessels together with a special microwave oven, MDS 2000, CEM, Düsseldorf, Germany. The amount of encapsulated contrast agent was calculated from the results obtained for total and unencapsulated solutes.

RESULTS

Variation of the Lipid Composition

The feasibility of producing small liposomes of various lipid compositions with the new continuous high pressure extrusion apparatus was tested using saturated and unsaturated as well as charged and uncharged lipids. The impact of the addition of different amounts of Chol to the phospholipid mixture was also investigated.

Irrespective of the lipid composition employed, extrusion was fast and easy, without congestion of polycarbonate membranes. Except for one preparation (DSPC) all mean diameters were well below 100 nm, as determined by PCS (TABLE 1). Variation coefficients smaller than 30 % indicated homogeneous size distributions for extruded liposome dispersions. Smallest mean diameters around 60 nm were achieved using the negatively charged phospholipids SPG and SPA in a molar concentration of 10 % in combination with SPC. Addition of up to 50 mol % Chol to SPC did not affect the extrusion process with regard to congestion of membranes, but led to a slight increase in mean diameters from 75 nm without Chol (SPC) to 93 nm with 50 mol % Chol (SPC:Chol 5:5). Liposomes composed of the saturated DSPC only, aggregated and eventually fused immediately after preparation resulting in a mean diameter of about 200 nm after 24 hours. The addition of Chol enabled the stabilisation of liposomes composed of saturated lipids. Thus, the mean diameter of a respective preparation (hydr.SPC:Chol 7:3) was only about 90 nm after 24 h.

Lipid Concentration

The ability of the new extrusion apparatus to cope with high lipid concentrations was investigated, using amounts between 50 and 400 mg SPC per g of preparation.

In all cases, 100 ml of MLV in tromethamine buffer (20 mM, pH 7.5) were extruded without clogging of the membranes (pore sizes: 5.0, 1.0, 0.4, 0.2, 0.1, 0.05 and 0.03 µm). At the highest lipid concentration (400 mg/g), however, maximum pressures of 10.5 MPa had to be employed, especially for passage through filters with 0.05 and 0.03 µm pore size in order to achieve reasonable flow rates. Although preparations at this concentration already tended to form gels, extrusion could still be performed without major difficulties. Mean vesicle diameters of liposomes increased slightly with rising lipid concentration from 64 nm at 50 mg/g lipid to 74 nm at 400 mg/g, whereas the CV was unaffected, displaying values well below 30 % .

TABLE 1

Mean Diameters of extruded Vesicles with various Lipid Compositions as determined by PCS

lipid composition (molar ratio)	mean diameter / variation coefficient (nm / %)
SPC	75 / 25.3
EPC	86 / 24.4
DSPC	201 / 48.5
SPC:Chol (9:1)	80 / 22.5
SPC:Chol (7:3)	83 / 21.7
SPC:Chol (5:5)	93 / 22.6
hydr.SPC:Chol (7:3)	96 / 22.0
SPC:SPE (9:1)	93 / 24.7
SPC:EPS (9:1)	78 / 23.1
SPC:SPG (9:1)	65 / 23.1
SPC:SPA (9:1)	66 / 21.2
SPC:SS (9:1)	90 / 21.1
SPC:DCP (9:1)	91 / 23.1
SPC:Chol:SPG (6:3:1)	79 / 22.8

Lipid concentration: 50 mg/g in tromethamine buffer (20 mM, pH 7.5). Sequential extrusion of MLV made by the film method, pore sizes: 5.0, 1.0, 0.4, 0.2, 0.1 and 0.05 µm. Batch size: 100 ml. (n=1)

Variation of final Pore Size

A variety of final pore sizes was employed during preparation of liposomes, in order to demonstrate the feasibility of the continuous high pressure extrusion method to generate vesicle populations with different mean diameters. MLVs were sequentially extruded through polycarbonate membranes with decreasing pore sizes until the desired final pore size was reached.

Smaller mean diameters of extruded vesicles were achieved by application of decreasing final pore sizes (FIGURE 2). Mean vesicle diameters varied from about 400 nm (5.0 µm final pore size) to 70 nm (0.03 µm final pore size). Along with falling mean diameters, heterogenei-

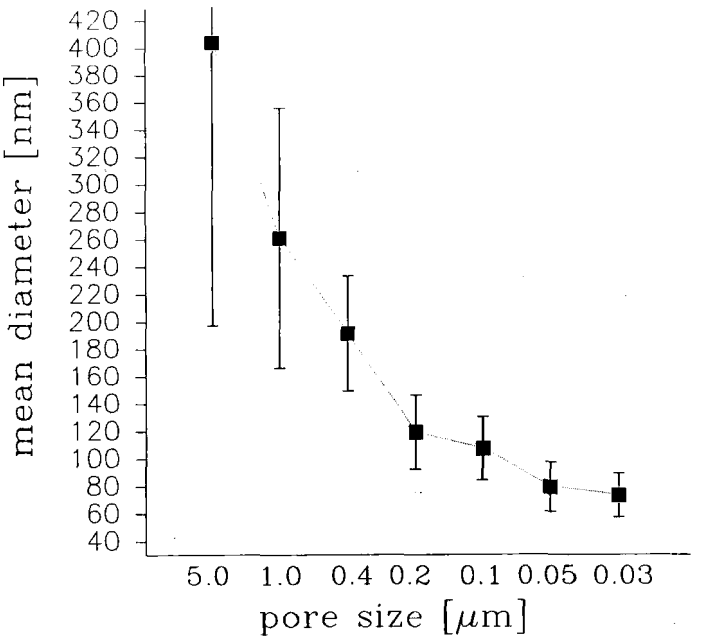

FIGURE 2

Mean diameter and standard deviation of extruded vesicles as obtained using different final pore sizes.
Lipid composition: SPC:Chol:SPG (6:3:1) in tromethamine buffer (20 mM, pH 7.5). Lipid concentration 50 mg/g. Batch size: 100 ml. (n=1)

ty of vesicle sizes, as indicated by the standard deviation (SD) of the population, decreased sharply. Thus the SD dropped from more than 200 nm for 5.0 μm extruded preparations to 16 nm for 0.03 μm.

Batch Size

For economic reasons, batch volumes of only 100 ml were usually used in this work. However, in order to show the potential of the new continuous high pressure extrusion apparatus in terms of upscaling, we prepared 1000 ml of an MLV suspension composed of SPC with a lipid concentration of 100 mg/g by direct dispersion of SPC in tromethamine buffer solution (20 mM, pH 7.5) using a high shear mixer (Ultraturrax, Ika,

Staufen, Germany). Extrusion was done with only three pore sizes: 1.0, 0.2 and 0.1 μm, applying 10 filter passages for each step.

During extrusion of the 1000 ml batch no clogging of membranes was observed and thus, maximum filtration flows of 500 ml/min could be obtained. The mean vesicle diameter in the final product as determined by PCS was 113 ± 34 nm, which correlates well with results obtained with a 100-ml batch size (compare FIGURE 2).

Encapsulation of Iopromide and Gd-DTPA

To investigate the trapping abilities of liposomes generated by the new continuous high pressure extrusion apparatus, vesicles encapsulating the two water-soluble contrast agents iopromide and Gd-DTPA were prepared. Extrusion was performed either with or without employment of three freeze-thaw cycles (after passage through 0.4 μm). Encapsulation efficiencies and mean vesicle diameters of contrast-carrying liposomes are shown in FIGURE 3.

The application of three freeze-thaw cycles greatly improved the encapsulation of iopromide, whereas for Gd-DTPA the effect was only minor. Entrapment values of contrast-carrying liposomes increased from 34.1 ± 1.2 % without freeze-thaw to 45.1 ± 1.9% for iopromide and from 44.2 ± 0.5 % to 49.5 ± 5.1 % for Gd-DTPA. Mean vesicle diameters remained almost unchanged, despite the employment of a freeze-thaw protocol.

Solute Concentration

In order to investigate the influence of the solute concentration on the encapsulation efficiency of liposomes made by the continuous high pressure extrusion apparatus, preparations with increasing contrast agent concentrations were produced.

Encapsulation efficiencies as summarized in FIGURES 4 and 5 markedly decreased with rising solute concentration. At initial iodine concentrations of 50 mg/g a maximum entrapment of 52.0 ± 1.4 % was achieved. Raising the iodine concentration to 200 mg/g caused a decrease of entrapment to 27.5 ± 1.8 % of total recovered iodine. For Gd-

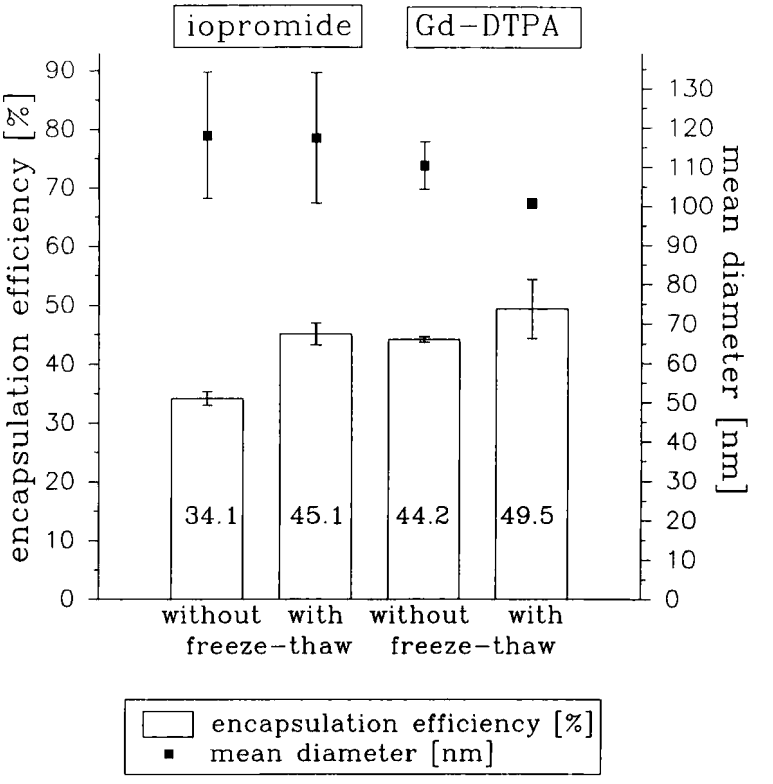

FIGURE 3

Influence of freeze-thaw on encapsulation efficiency of iopromide- and Gd-DTPA-carrying liposomes.
Lipid composition: SPC:Chol:SPG (6:3:1) for iopromide, SPC:Chol (7:3) for Gd-DTPA. Lipid concentration 160 mg/g for iopromide, 150 mg/g for Gd-DTPA. Iodine concentration 100 mg/g, Gd concentration 180 µmol/g. Sequential extrusion of MLV made by the film method, pore sizes: 5.0, 1.0, 0.4, 0.2 and 0.1 µm. Batch size: 100 ml. (error bars denote SDs, n=3)

DTPA the decline was even sharper. Trapping efficiencies dropped from 63.7 ± 3.4 % at 90 µmol/g Gd to 22.4 ± 0.9 % at 360 µmol/g Gd concentration. Solute concentration had no pronounced effect on mean diameters of liposome preparations, which ranged from 100 to 120 nm.

Reproducibility

Six batches of iopromide-carrying liposomes were produced under identical conditions, to test the ability of the new continuous high pressure

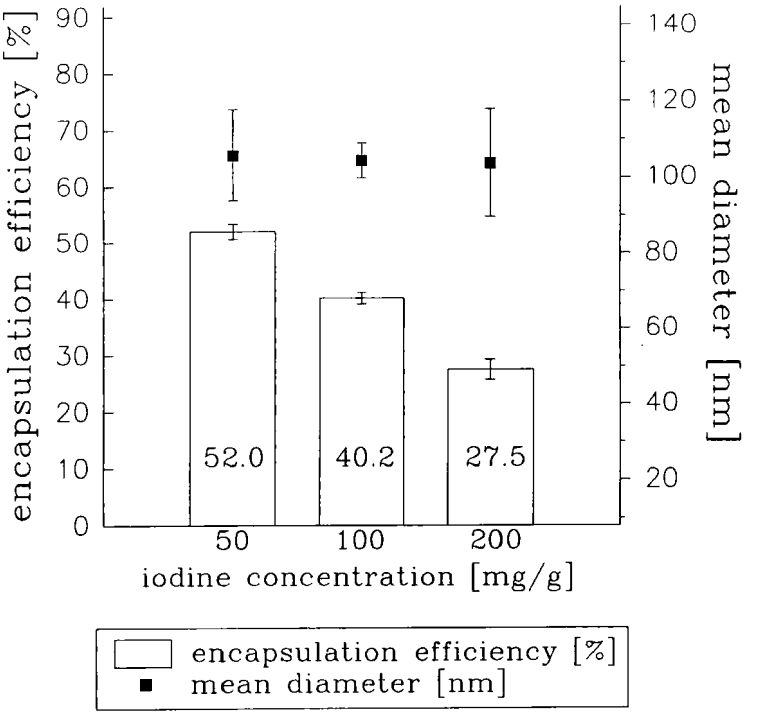

FIGURE 4

Encapsulation efficiency and mean diameter of extruded iopromide-carrying liposomes in terms of the initial contrast agent concentration.
Lipid composition: SPC:Chol:SPG (6:3:1). Lipid concentration 150 mg/g. Sequential extrusion of MLV made by the film method, pore sizes: 5.0, 1.0, 0.4, 0.2 and 0.1 µm. 3 freeze-thaw cycles. Batch size: 100 ml. (error bars denote SDs, n=3)

extrusion apparatus to generate liposomes with reproducible encapsulation efficiency and vesicle size.

As can be seen in TABLE 2, both features showed only minor fluctuations. Entrapment ranged from 39.6 to 41.4 % and mean diameters from 99 to 122 nm. The average of all preparations was a value of 40.4 ± 0.9 % for the encapsulation efficiency and 111 ± 8.7 nm for the mean diameter.

Stability

Void, as well as iopromide- and Gd-DTPA-carrying liposomes were tested for their storage stability at 2-8°C. For each contrast agent the

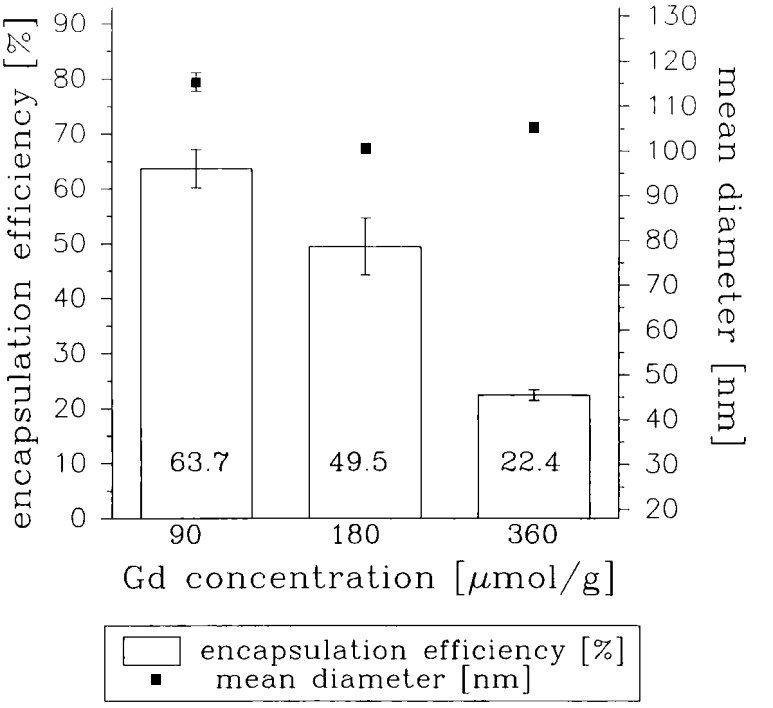

FIGURE 5

Encapsulation efficiency and mean diameter of extruded Gd-DTPA-carrying liposomes in terms of the initial contrast agent concentration.
Lipid composition: SPC:Chol (7:3). Lipid concentration 150 mg/g. Sequential extrusion of MLV made by the film method, pore sizes: 5.0, 1.0, 0.4, 0.2 and 0.1 μm. 3 freeze-thaw cycles. Batch size: 100 ml. (error bars denote SDs, n=3)

encapsulation efficiencies and vesicle sizes of three batches, prepared under identical conditions, were measured prior to storage and after 6 months (TABLE 3). Additionally, the mean diameters of void vesicles with three different lipid compositions obtained after storage for 18 months at 2-8° C were compared to those measured right after preparation (TABLE 4).

The encapsulation efficiencies of iopromide- and Gd-DTPA-carrying vesicles were unaffected by storage. In all cases, however, average vesicle diameters slightly increased, from 104 (average CV 32.9 %) to

TABLE 2

Reproducibility of the Continuous High Pressure Extrusion Method regarding the Encapsulation Efficiency and Vesicle Size of Iopromide-carrying Liposomes.

batch	encapsulation efficiency [%]	mean diameter [nm]
1	39.6	99
2	41.4	107
3	39.5	107
4	39.8	120
5	41.4	122
6	40.7	109
average ± SD	**40.4 ± 0.9**	**111 ± 8.7**

Lipid composition: SPC:Chol:SPG (6:3:1). Lipid concentration: 150 mg/g. Iodine concentration 100 mg/g. Sequential extrusion of MLV made by the film method, pore sizes: 5.0, 1.0, 0.4, 0.2 and 0.1 µm. 3 freeze-thaw cycles. Batch size: 100 ml.

123 nm (average CV 33.3 %) for iopromide and from 101 (average CV 31.4 %) to 112 nm (average CV 29.9 %) for Gd-DTPA. Mean diameters of void vesicles did not change even after storage for 18 months. Only the heterogeneity of vesicle size distributions slightly increased, as indicated by growing CVs.

DISCUSSION

The production of liposomes of variable size using the new high pressure extrusion apparatus proved to be fast and simple. Its design permits the repetitive passage of preparations through filters, without opening a pressure chamber, which is necessary for discontinuous extruders. Thus, recirculation (5 times at each pore size) was performed in a time-saving manner, simply by refilling the open supply vessel.

TABLE 3

Long-term Stability of Iopromide- and Gd-DTPA-carrying Liposomes (Storage at 2-8° C for 6 Months)

batch	encaps. eff. after preparation [%]	encaps. eff. after 6 months [%]	mean Diam./CV after preparation [nm/%]	mean Diam./CV after 6 months [nm/%]
iopromide				
a	39.6	41.4	99/32.3	115/32.2
b	41.4	41.8	107/30.3	132/36.7
c	39.5	41.1	107/36.2	123/31.0
mean	**40.2 ± 1.1**	**41.4 ± 0.4**	**104/32.9**	**123/33.3**
Gd-DTPA				
a	43.7	43.7	100/32.6	109/31.5
b	53.6	54.4	102/30.0	111/28.0
c	51.0	51.4	100/30.4	117/30.2
mean	**49.4 ± 5.1**	**49.8 ± 5.5**	**101/31.4**	**112/29.9**

Lipid composition: SPC:Chol:SPG (6:3:1) for iopromide, SPC:Chol (7:3) for Gd-DTPA. Lipid concentration: 150 mg/g. Iodine concentration: 100 mg/g, Gd concentration: 180 µmol/g. Sequential extrusion of MLV made by the film method, pore sizes: 5.0, 1.0, 0.4, 0.2 and 0.1 µm. 3 freeze-thaw cycles. Batch size: 100 ml.

TABLE 4

Vesicle Size of void Vesicles prior to and after Storage at 2-8° C for 18 Months

lipid composition	meanDiam./CV after preparation [nm/%]	mean diam./CV after 18 months [nm/%]
SPC:Chol (9:1)	68/23.5	67/27.9
SPC:EPS (9:1)	61/23.0	60/33.2
SPC:SPG (9:1)	60/21.7	61/33.5

Lipid concentration: 50 mg/g in 20 mM tromethamine buffer, pH 7.5. Sequential extrusion of MLV made by the film method, pore sizes: 5.0, 1.0, 0.4, 0.2 , 0.1, 0.05 and 0.03 µm. Batch size: 100 ml. (n=1)

Another option, which we did not use in the present study, is the continuous recirculation of liposomes for a desired time. Exchange of polycarbonate membranes in the high pressure filter holder was also very fast and easy.

The suitability of the apparatus for the extrusion of liposomes of various lipid compositions was demonstrated, using saturated and unsaturated as well as charged and uncharged lipids and Chol in different molar ratios. Almost all lipid compositions led to mean vesicle diameters well below 100 nm with homogeneous size distributions (CV < 30 %), when a final pore size of 0.05 μm was employed. Only liposomes composed of saturated lipids showed larger diameters due to aggregation and eventually fusion after cooling below the phase transition temperature, a phenomenon already described for such lipids [7, 11].

The extrusion of preparations with extremely high lipid concentrations up to 400 mg/g did not cause any difficulties regarding congestion of polycarbonate membranes. The application of high pressures, up to 10.5 MPa, enabled the fast passage of preparations even through pores with diameters as small as 0.03 μm. By choosing an appropriate filter holder construction, a further increase in maximum pressure and, thus, an improvement in flow rates at high lipid concentrations should be an attainable goal.

One major advantage of the extrusion method in general is its ability to produce liposomes of defined mean diameters within certain limits. Varying the pore size during the final extrusion step between 0.03 and 5.0 μm, we obtained preparations with a whole range of different mean diameters between 70 and 400 nm. The continuous high pressure extrusion method thus allows a rational modification of liposome size.

The volumes of the batches extruded in this work did not usually exceed 100 ml. However, we were able to demonstrate that the production of batch sizes on the liter scale with the new apparatus is possible without any problems. Due to the high maximum pressure and the structural design, flows up to 500 ml/min were achieved. Thus, 1000 ml of liposomal preparation were passed through the filters within just a few minutes, even when small pore sizes of 0.1 μm were used. The supply of sufficient

amounts of extruded liposomes for toxicological and clinical trials as well as a further upscale to industrial level should now be easy to accomplish.

Limited batch size remains a major disadvantage of currently available extruders. These discontinuous devices usually have capacities of 50 ml or less [4, 7, 8], because they work with metal vessels, which must not exceed a certain volume, in order to withstand high pressures. To our knowledge, the only apparatus described so far which was able to work on a liter scale [6], could apply maximum pressures of about 1 MPa, only one tenth of the value we achieved. With such an extruder, clogging of polycarbonate membranes can hardly be avoided when high lipid concentrations or lipids with higher phase transition temperatures are used. Besides that, only small filter flows can be reached with low pressures. The construction of large metal vessels with volumes around or above 1 l, that can withstand high pressures of 10 MPa or more, is very complicated and expensive. Therefore, the concept of continuously forcing liposomal preparations through a high pressure filter holder by means of a gas-driven pump represents a breakthrough in the development of large-scale extrusion methods.

Investigation of the trapping properties of continuously extruded liposomes, which contained the water-soluble contrast agents iopromide and Gd-DTPA as model substances, revealed quite high encapsulation efficiencies. Without employing a freeze-thaw technique we were able to reach entrapment values as high as 34 % for iopromide and 44 % for Gd-DTPA. The application of three freeze-thaw cycles further increased the encapsulation of the preparations to 45 % and 49.5 %, respectively. Several possible explanations for the observed improvement of the entrapment of freeze-thawed liposomes have already been proposed, one being that small liposomes in a preparation will be forced to fuse and form vesicles with increased diameters, which are able to encapsulate larger volumes of the aqueous phase [5]. Additionally, growing distances between the bilayers of multi- and oligolamellar vesicles and a decrease in overall lamellarity of the system after the freeze-thaw protocol have been suggested as explanations for this phenomenon [4, 12]. Preliminary results of negative staining transmission electron microscopy obtained in our laboratory (data not shown), however, seem to confirm the former mechanism.

Applying low contrast agent concentrations (50 mg/g iodine and 90 µmol/g Gd), we obtained maximum encapsulation efficiencies of freeze-thawed preparations of more than 50 % for iopromide and over 60 % for Gd-DTPA, although the mean diameters of these liposome preparations were only around 100 nm. To our knowledge, these results exceed the highest encapsulation efficiencies published so far for passive entrapment of water-soluble contrast agents [13, 14]. An increase in the solute concentration led to a sharp decline in entrapment values for both substances. High viscosity and osmotic pressures are believed to somehow disturb the formation of vesicles and thus deteriorate the entrapment [15, 16].

The ability of the new continuous high pressure extrusion method to produce liposomes with constant batch-to-batch quality was found to be very good. Encapsulation efficiencies and mean diameters of 6 batches prepared under identical conditions hardly differed at all. The reproducibility of production methods represents one of the most important features for the successful development of liposomal preparations.

Long term stability testing of continuously extruded liposomes also yielded satisfactory results. Encapsulation efficiencies of contrast-carrying vesicles remained almost constant after 6 months' storage at 2-8° C. Only the vesicle size of liposomal preparations changed slightly. The small increase in size is not, however, expected to alter the biodistribution pattern of these vesicles significantly.

CONCLUSION

The continuous high pressure extrusion method, introduced in this work, proved to be suitable for the fast and reproducible generation of stable liposomal preparations with a variety of lipid compositions. Large batches up to the liter scale with maximum lipid concentrations of 400 mg/g could be extruded with flows as high as 500 ml/min, when maximum pressures of 10.5 MPa were employed. Polycarbonate membranes with pore sizes ranging from 0.03 to 5.0 µm yielded defined mean vesicle diameters between 60 and 400 nm. Contrast-carrying liposomes displayed encapsulation efficiencies of over 50 % for iopromide and more

than 60 % for Gd-DTPA. The observed stability of continuously extruded vesicles appears to allow unrestricted application of these preparations after storage of at least 6 months.

The new concept of the extruder, based on the idea of continuously forcing liposomal suspensions through membranes embedded in a high pressure filter holder by means of a gas-driven pump, represents a major step forward in the development of liposome production on an industrial scale.

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