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Generation of contrast-carrying liposomes of defined size with a new continuous high pressure extrusion method

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Abstract

A novel continuous high pressure extrusion method was evaluated for the generation of radiopaque and paramagnetic liposomes. The magnetic resonance contrast agent gadolinium diethylenetriaminepentaacetic acid (Gd-DTPA) as well as the X-ray contrast agent iopromide were used as water-soluble model substances for liposomal encapsulation. The continuous process, which is introduced here, allows the fast and efficient extrusion of large batches of liposomal preparations with maximum flow rates of 500 ml/min. Applying high pressures up to 10.5 MPa, MLV prepared by the film method were sequentially extruded through polycarbonate membranes of decreasing pore size. Encapsulation efficiency was found to be dependent on lipid composition and concentration, amount of contrast agent in the preparation as well as choice of final pore size for extrusion. Application of freeze-thaw cycles markedly improved the entrapment of iopromide, whereas for Gd-DTPA freeze-thaw surprisingly turned out to have only minor effects. Entrapment values rose with increasing lipid concentration and fell sharply with increasing solute concentration. Mean liposome diameters could be varied using polycarbonate membranes of differing pore sizes. Smaller final pore sizes led to vesicle populations with smaller mean diameters and lower encapsulation efficiencies. Excellent maximum encapsulation efficiencies of more than 50% for iopromide and over 60% for Gd-DTPA were obtained for vesicles with mean diameters of around 100 nm, as determined by photon correlation spectroscopy (PCS) and confirmed by negative-staining electron microscopy. Employing medium contrast (100 mg/g iodine and 180 μmol/g Gd) and lipid concentrations (150 mg/g), entrapment values as high as 40% for the X-ray and 50% for the paramagnetic contrast agent could still be achieved. Best results were obtained using a lipid mixture of soy phosphatidylcholine (SPC), cholesterol (Chol) and soy phosphatidylglycerol (SPG) in a molar ratio of 6:3:1 for iopromide and SPC, Chol 7:3 for Gd-DTPA. Liposomal preparations remained stable upon storage at 2–8°C for 6 months. The new continuous high pressure extrusion method proved to be suitable for the generation of large volumes of stable, contrast-carrying liposomes with outstanding encapsulation efficiencies.

Keywords: Liposome; Contrast agent; Gd-DTPA; Iopromide; Extrusion; Continuous process; High pressure; Encapsulation efficiency

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1. Introduction

Magnetic resonance tomography (MRT) and computerized tomography (CT) have become important tools in diagnostic imaging. With the introduction of modern contrast agents, for example, the paramagnetic gadolinium diethylenetriaminepentaacetic acid (Gd-DTPA) for MRT or the non-ionic iodinated benzoic acid derivative iopromide for CT, these diagnostic methods could be further improved. Despite their totally different mechanism of providing contrast, water-soluble diagnostics like Gd-DTPA and iopromide have one characteristic feature in common: after intravenous injection, they extravasate within minutes and are rapidly cleared by the kidneys (Weinmann et al., 1984; Seltzer, 1989). For several diagnostic purposes, however, slower clearance and longer blood half-lives of contrast agents are needed. A prolonged circulation time could be of great advantage, especially for use as blood pool agents in perfusion studies or for the discrimination of tumors from healthy tissues due to differences in contrast agent uptake.

A suitable way to alter the biodistribution pattern of water-soluble contrast agents is their encapsulation in liposomes, small lipid vesicles, ranging from 30 nm to several μm in size. Liposomes consist of one (unilamellar) or several (oligo- and multilamellar) bilayers, which form spontaneously, when amphiphilic lipids like phosphatidylcholine are dispersed in water. During the formation of closed, spheric structures, solutes can be entrapped within the aqueous core and also in the interlamellar space of multilamellar vesicles. Liposomes, which are usually too large to extravasate, are predominantly removed from the circulation by phagocytic cells of the RES, thus accumulating to a great extent in organs like liver and spleen. This naturally occurring biodistribution pattern can be used for passive targeting of diagnostics to these organs (e.g., Sachse et al., 1993).

A variety of methods has been suggested in the past for the laboratory-scale production of contrast-carrying liposomes (Ryan et al., 1983; Caride et al., 1984; Grant et al., 1987; Seltzer et al., 1988; Henze et al., 1989; Fritz et al., 1991). Lately, the so-called extrusion method (Olson et al., 1979), where suspensions of multilamellar vesicles (MLV) are passed through filters with straight pores of defined size, has gained increasing attention in this field (Tilcock et al., 1989; Unger et al., 1991). By choosing filters with appropriate pore sizes, liposomes with defined diameters can be produced within certain limits (Olson et al., 1979; Bally et al., 1988; Nayar et al., 1989). Besides the possibility to influence mean liposome diameter, the extrusion method bears several additional advantages: (1) It yields quite homogeneous size distributions (Olson et al., 1979; Mayer et al., 1986); (2) there are no solvents or detergents to be removed from the final product (Hope et al., 1985); and (3) a variety of different lipids can be used (Hope et al., 1985; Nayar et al., 1989).

Since extrusion at low pressures (≤ 1 MPa, e.g., Amselem et al., 1990) is restricted to relatively low lipid concentrations and to certain lipid mixtures, extrusion devices with maximum pressures around or above 5 MPa have recently gained increasing interest (Mayer et al., 1986; Amselem et al., 1993). The limited volume capacity (e.g., often below 50 ml (Mayer et al., 1986; Nayar et al., 1989)) together with sometimes insufficient maximum pressure, however, remain the main disadvantages of most currently available discontinuous extruders. The resulting inability to generate large batches for the supply of toxicological and clinical trials has been the major obstacle on the way to a wide use of the filter extrusion method for liposome production.

To overcome these problems, we have designed and tested a new continuous extrusion apparatus which operates at high pressures of up to 10.5 MPa. This apparatus allows continuous and efficient extrusion at maximum flow rates of 500 ml/min (depending on lipid composition and concentration as well as filter pore size). Our aim was to produce larger batches (≥ 100 ml) of contrast-carrying vesicles with diameters around 100 nm. We chose this vesicle size, because larger liposomes (≥ 200 nm) have been reported to be removed by the RES faster than smaller ones (Woodle and Lasic, 1992) and since we wanted to obtain prolonged circulation half-lives. Iopromide and Gd-DTPA were used as model substances in

order to evaluate the potential of the new method to encapsulate water-soluble contrast agents. Investigating the factors affecting the encapsulation efficiency, size and storage stability of continuously extruded vesicles, such as charge, contrast agent and lipid concentration as well as the choice of final pore size of the employed filters, we were able to optimize liposomal preparations achieving high encapsulation efficiencies and excellent long-term storage stability.

2. Materials and methods

2.1. Lipids and contrast agents

Soy phosphatidylcholine (Lipoid S100, SPC) and soy phosphatidylglycerol (Lipoid SPG, SPG) were from Lipoid KG, Ludwigshafen, Germany. Dicetyl phosphate (DCP) and cholesteryl hemisuccinate (CholHS) were obtained from Sigma Chemical Co., St. Louis, MO, U.S.A. Cholesterol (Chol) was from Solvay Duphar B.V., Veenendaal, The Netherlands. Stearylamine (SA) was from Fluka Chemie AG, Buchs, Switzerland. All lipids were used without further purification.

Ultravist® 370, containing the non-ionic, water-soluble X-ray contrast agent iopromide (Mol. Wt 791.14) was obtained from Schering AG, Berlin, Germany, as well as Magnevist®, containing the ionic, water-soluble paramagnetic gadolinium diethylenetriaminepentaacetic acid (Gd-DTPA) as its dimeglumine salt (Mol. Wt 938.01). Contrast agent solutions were diluted to the desired concentration with either 20 mM tromethamine buffer, pH 7.5 (Ultravist®) or bidistilled water (Magnevist®).

2.2. Preparation of contrast-carrying liposomes

Lipid mixtures were dissolved in ethanol (96% (v/v), reagent grade, obtained from E. Merck, Darmstadt, Germany) at elevated temperatures (50–60°C). By complete removal of the solvent during rotary evaporation a thin lipid film was deposited on the walls of a round bottom flask. Multilamellar vesicles (MLV) were generated by dissolving the lipid film at room temperature with

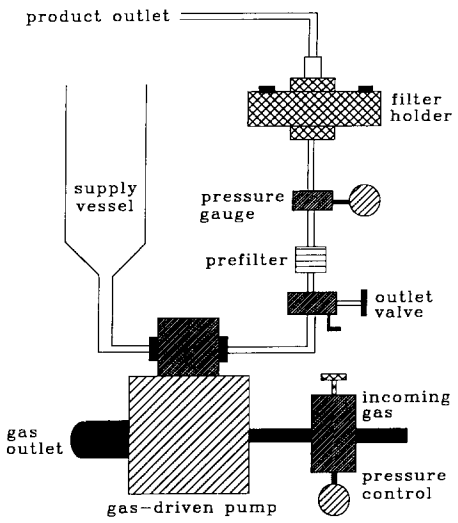

Fig. 1. Schematic diagram of the continuous high pressure extrusion apparatus.

contrast agent solution. Employed lipid concentrations were 150–160 mg/g and contrast agent concentrations 100 mg/g iodine and 180 μmol/g Gd, except where stated otherwise.

For the subsequent extrusion of MLV dispersions we used the newly developed continuous high pressure extrusion apparatus Maximator® Model HPE 10.0–250, Schmidt, Kranz & Co., Zorge, Germany. The structural design of this device (Fig. 1) allows the fast (flow rates up to 500 ml/min), continuous extrusion of liposome suspensions at pressures up to 10.5 MPa. Liposomal dispersions (batch sizes in this study ranged from 100 to 200 ml) were forced five times through two stacked polycarbonate membranes (Nuclepore GmbH, Tübingen, Germany). To obtain vesicles with mean diameters around 100 nm, we used filters with decreasing pore sizes of 5.0, 1.0, 0.4, 0.2 and 0.1 μm, employing a total of 25 passages for one extrusion protocol. Usually, after passage through membranes with 0.4 μm pore size and before continuing further extrusion, three freeze-thaw cycles were carried out. Liposomes were frozen in glass vials at −70°C in methanol/CO_2 and thawed at 70°C in a water bath. For those experiments where the final pore size was above 0.2 μm the freeze-thaw procedure was

already carried out after extrusion through 5.0 μm. Following the final extrusion step, liposomes were filtered through microbe retentive filters (cellulose acetate, pore size 0.22 μm, Satorius, Göttingen, Germany) and filled into sterile glass vials under aseptic conditions.

2.3. Liposome size

Liposome size was determined by photon correlation spectroscopy (PCS) using a Submicron Particle-Sizer Autodilute®, Model 370, Nicomp Instrument Corp., Santa Barbara, CA, U.S.A. Calculation of vesicle size distributions was carried out in the volume-weighted Gaussian analysis modus following 10 min of measurement at an angle of 90° and a temperature of 25°C.

For comparison, selected liposomal preparations were also characterized using negative staining transmission electron microscopy (TEM, Phillips EM 400, Phillips AG, Eindhoven, The Netherlands). Negative staining was carried out with a 2.0% uranyl acetate solution. Mean diameters were calculated from the micrographs using a correction factor of 0.75 as described elsewhere (Olson et al., 1979).

2.4. 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 throughout the text, 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 total contrast agent concentration, liposomes had to be dissolved. For iopromide analysis this was done by dilution of small aliquots of liposomal preparation with methanol. Gd-DTPA liposomes were destroyed by application of high pressures and elevated temperatures in 32% HNO_3 using high pressure vessels in 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.

2.5. Contrast agent to lipid ratio

To calculate the contrast agent to lipid ratio, the concentration of the unsaturated SPC was measured by high-performance liquid chromatography (HPLC) with a mixture of methanol, acetonitrile, water and conc. H_3PO_4 (1000:1000:100:1 volume ratio, all solvents spectroscopic grade and from E. Merck, Darmstadt, Germany) as the mobile phase and a 250 mm Lichrosorp-NH_2 column, Knauer, Berlin, Germany, as the stationary phase. Flow rate was 1.0 ml/min and the detection wavelength was set to 205 nm. After analysis of phospholipid, total lipid concentration was calculated using the factor of the molar portion of SPC in the lipid mixture. The amount of encapsulated contrast agent, expressed either as amount of Gd-DTPA dimeglumine salt or iodine, was then divided by the calculated total lipid weight in the preparation to give the respective contrast agent to lipid ratio.

2.6. Zeta potential

The zeta potential of void vesicles was determined after measurement of the electrophoretic mobility (20 s at 25°C), using a Zetasizer IIc, Malvern Ltd, Malvern, U.K.

3. Results

3.1. Variation of final pore size

Employing different final pore sizes during extrusion, we tested the feasibility of the new device for size reduction and variation of the mean diameter of liposomal preparations. Extrusion at all pore sizes was fast and easy, without congestion of polycarbonate membranes. As ex-

pected, the application of smaller pore sizes resulted in vesicle populations with smaller mean diameters and decreased entrapment values. For iopromide-carrying vesicles (SPC : Chol : SPG 6 : 3 : 1) we used five different final pore sizes, 1.0, 0.4, 0.2, 0.1 and 0.05 μm, and reduced the mean diameter of extruded vesicle populations from 211 $\pm$ 2 to 87 $\pm$ 2 nm. Consequently, the encapsulation efficiency fell from 50.6 $\pm$ 1.5 to 34.2 $\pm$ 0.5% (Fig. 2). In the case of Gd-DTPA (SPC : Chol 7 : 3) only three different final pore sizes, 0.2, 0.1 and 0.05 μm were used and mean diameters decreased from 158 $\pm$ 3 nm for 0.2 μm pore size to 84 $\pm$ 3 nm for 0.05 μm. At the same time, the entrapment value dropped from 50.1 $\pm$ 2.8 to 43.2 $\pm$ 0.4% (Fig. 2).

3.2. Lipid concentration

Raising the lipid concentration of iopromide preparations composed of SPC : Chol : SPG (6 : 3 : 1) from 50 to 150 mg/g led to a marked increase in encapsulation efficiency from 19.3 $\pm$ 1.2 to 40.2 $\pm$ 1.1% (Fig. 3). The highest lipid concentration used here was 160 mg/g with entrapment values of 45.1 $\pm$ 1.9%. Above this concentration, liposome suspensions tended to form viscous gels, which were difficult to extrude. Gd-DTPA preparations composed of SPC : Chol (7 : 3) were produced with lipid concentrations between 100 and 250 mg/g. Concentrations above 250 mg/g again led to gel formation. Encapsulation efficiencies (Fig. 4) ranged from 37.9 $\pm$ 0.8% (100 mg/g lipid) to 63.2 $\pm$ 0.3% (250 mg/g lipid).

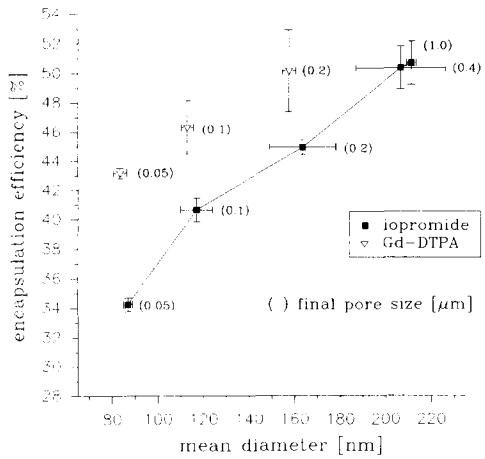

Fig. 2. Contrast-carrying liposomes prepared with different final pore sizes: Influence of vesicle size on encapsulation efficiency. Lipid composition: SPC : Chol : SPG (6 : 3 : 1) for iopromide; SPC : Chol (7 : 3) for Gd-DTPA. Lipid concentration, 150 mg/g; iodine concentration, 100 mg/g; Gd concentration, 180 μmol/g; three freeze-thaw cycles (error bars denote SD, $n = 3$).

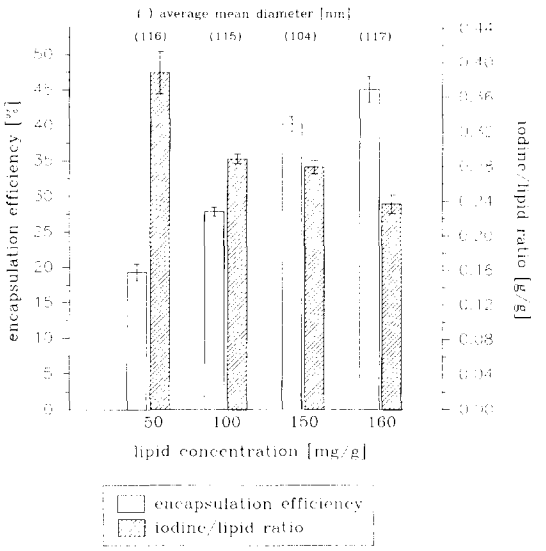

Fig. 3. Influence of lipid concentration on encapsulation efficiencies and iodine/lipid ratio of iopromide-carrying liposomes. Lipid composition: SPC : Chol : SPG (6 : 3 : 1). Iodine concentration, 100 mg/g; three freeze-thaw cycles; final pore size, 0.1 μm (error bars denote SD, $n = 3$).

Increasing the lipid concentration, however, led to a decrease in the contrast agent to lipid ratio in both cases (Fig. 3 and 4). The iodine to lipid ratio fell from 0.39 $\pm$ 0.03 (50 mg/g lipid) to 0.24 $\pm$ 0.01 (160 mg/g lipid) and the Gd-DTPA-dimeglumine salt to lipid ratio dropped from 0.65 $\pm$ 0.02 (100 mg/g lipid) to 0.42 $\pm$ 0.02 (250 mg/g lipid).

The lipid concentration had no effect on the mean diameter of the liposomal suspensions. In all cases diameters ranged from 100 to 120 nm.

3.3. Contrast agent concentration

Encapsulation efficiencies of both iopromide and Gd-DTPA liposomes dropped with rising

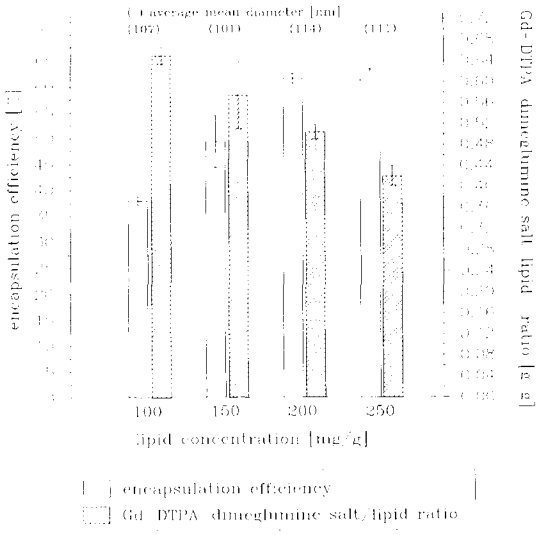

Fig. 4. Influence of lipid concentration on encapsulation efficiencies and Gd-DTPA-dimeglumine salt/lipid ratio of Gd-DTPA-carrying liposomes. Lipid composition: SPC:Chol (7:3). Gd concentration, 180 μmol/g; three freeze-thaw cycles; final pore size, 0.1 μm (error bars denote SD, $n = 3$).

contrast agent concentration (Fig. 5). While an encapsulation efficiency as high as $52.0 \pm 1.4\%$ was achieved using an iodine concentration of 50 mg/g, only $27.5 \pm 1.8\%$ of total contrast agent could be entrapped at iodine concentrations of 200 mg/g. Gd-DTPA entrapment fell from $63.7 \pm 3.4\%$ at a Gd concentration of 90 μmol/g to $22.4 \pm 0.9\%$ at 360 μmol/g. Contrast agent concentration had no effect on the mean diameter of the vesicles, which were again between 100 and 120 nm.

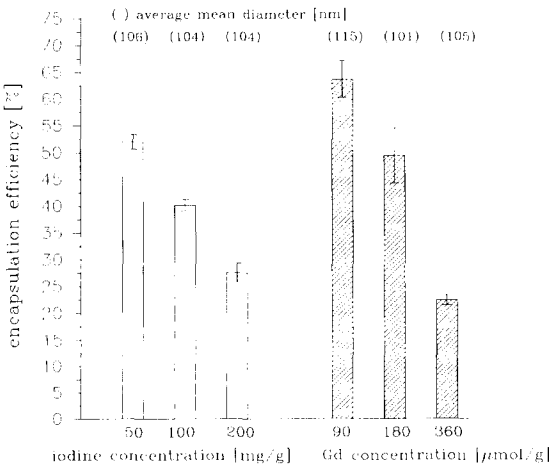

Fig. 5. Influence of contrast agent concentration on encapsulation efficiencies of liposomes. Lipid composition: SPC:Chol:SPG (6:3:1) for iopromide; SPC:Chol (7:3) for Gd-DTPA. Lipid concentration, 150 mg/g; three freeze-thaw cycles; final pore size, 0.1 μm (error bars denote SD, $n = 3$).

3.4. Freeze-thaw cycles

The application of three freeze-thaw cycles greatly improved the encapsulation of iopromide while in the case of Gd-DTPA the effect was only small. Entrapment values rose from $34.1 \pm 1.2\%$ without freeze-thaw to $45.1 \pm 1.9\%$ for iopromide (lipid composition: SPC:Chol:SPG 6:3:1, lipid concentration 160 mg/g) and from 44.2 ± 0.5 to $49.5 \pm 5.1\%$ for Gd-DTPA (lipid composition: SPC:Chol 7:3, lipid concentration 150 mg/g). Differences in mean diameters caused by freeze-thaw could not be detected. In all cases, they ranged from 100 to 120 nm. Because of its positive influence on encapsulation, freeze-thaw was generally applied throughout all experiments.

3.5. Lipid composition: use of charged lipids

The addition of the negatively charged lipids DCP and SPG led to increasing encapsulation efficiencies for iopromide compared to the neutral preparations, whereas CholHS showed no effect (Fig. 6). Thus, $27.6 \pm 0.1\%$ of the X-ray contrast agent was entrapped in the neutral formulations composed of SPC:Chol (7:3), while 40.7 ± 1.5 and $40.8 \pm 1.6\%$ were entrapped after addition of 10% SPG and DCP, respectively. In contrast, the use of charged lipids had no significant effect on the encapsulation of Gd-DTPA (Fig. 6). With the neutral composition SPC:Chol (7:3), however, encapsulation efficiency already amounted to $49.5 \pm 5.1\%$.

The use of DCP consistently led to a congestion of the polycarbonate membranes with pore sizes smaller than 0.2 μm so that the last extrusion step (0.1 μm) could not be performed for DCP liposomes. Therefore, these preparations displayed larger vesicle sizes than the other lipo-

some suspensions, which had mean diameters around 100 nm as determined by PCS.

While all negatively charged formulations containing iopromide remained macroscopically stable after storage for 6 months at 2–8°C, the neutral liposomes tended to aggregate so that small amounts of precipitate were observed. In the case of Gd-DTPA only those preparations composed of SPC:Chol (7:3) and SPC:Chol:CholHS (6:3:1) remained stable, whereas DCP and SPG caused aggregation of liposomes.

Thus, optimum formulations regarding encapsulation and macroscopic stability used throughout this study, were SPC:Chol:SPG (6:3:1) for iopromide and SPC:Chol (7:3) for Gd-DTPA.

For further investigation of the influence of charged lipids on vesicle properties, the zeta potential of void vesicles (50 mg/g total lipid, SPC:Chol 7:3 or SPC:Chol:Charged Lipid 6:3:1, final pore size for extrusion 0.4 μm, $n = 1$) containing 10 mol% of charged lipids were determined and compared to a neutral preparation. Using DCP and SPG, zeta potentials of −53.7 and −55.5 mV, respectively, were achieved, whereas employment of the same molar amount of CholHS only resulted in a zeta potential of −46.3 mV. The neutral formulation displayed a value of −4.3 mV.

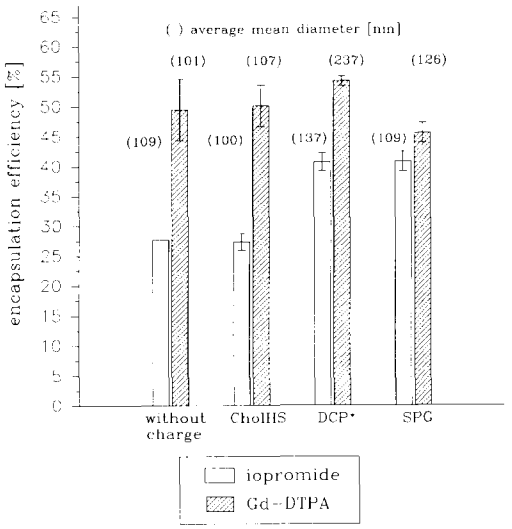

Fig. 6. Influence of charged lipids (10% molar) on encapsulation efficiencies of liposomes. Lipid composition: SPC:Chol (7:3) or SPC:Chol:charged lipid (6:3:1). Lipid concentration 160 mg/g for iopromide; 150 mg/g for Gd-DTPA; iodine concentration, 100 mg/g; Gd concentration, 180 μmol/g; three freeze-thaw cycles; final pore size, 0.1 μm (*final pore size 0.2 μm) (error bars denote SD, $n = 3$).

Table 1
Long-term stability of iopromide- and Gd-DTPA-carrying liposomes (storage at 2–8°C for 6 months)

Batch	Encapsulation efficiency (%)		Mean diameter/CV (nm/%)	
	After preparation	After 6 months	After preparation	After 6 months
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
Average	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
Average	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; final pore size, 0.1 μm; three freeze-thaw cycles.

3.6. Storage stability at 2–8°C

For stability tesing, liposomes produced as mentioned in Table 1 were stored at 2–8°C without prior removal of the free drug. After 6 months, none of the preparations showed visible inhomogeneities (sedimentation). Encapsulation was also unaffected by storage. The average vesicle size of the preparations, however, slightly increased from 104 to 123 nm for iopromide and from 101 to 112 for Gd-DTPA (Table 1).

3.7. PCS and TEM data

In order to evaluate the quality of the liposome size distributions measured by PCS, we also determined vesicle sizes of three liposomal preparations by means of negative staining TEM. The comparison of PCS and TEM revealed only slight differences between the results of the two methods. In all three preparations, however, di-

Table 2
Comparison of mean diameter and coefficient of variation (CV) of three different liposomal preparations determined by PCS and TEM

Production method	Entrapped contrast agent	Mean diameter /CV (nm/%)	
		TEM	PCS
Freeze-thaw extrusion [a]	iopromide	94/31.3	112/32.5
Freeze-thaw extrusion [b]	Gd-DTPA	85/30.2	105/30.9
Extrusion without Freeze-thaw [b]	Gd-DTPA	78/35.6	101/26.8

[a] Lipid composition: SPC:Chol:SPG (6:3:1). Lipid concentration, 150 mg/g; iodine concentration, 100 mg/g; final pore size, 0.1 μm ($n = 1$).
[b] Lipid composition: SPC:Chol (7:3). Lipid concentration, 150 mg/g; Gd concentration, 180 μmol/g; final pore size, 0.1 μm ($n = 1$).

ameters determined by PCS were larger than those obtained from TEM (Table 2). Thus, for example, the mean diameter of freeze-thawed and extruded Gd-DTPA liposomes obtained from TEM, was found to be 85 nm, as compared to 105 nm (PCS). TEM, however, confirmed that the size distributions of extruded vesicles were unimodal and showed almost symmetrical form (Fig. 7). This is in good correlation with our PCS data,

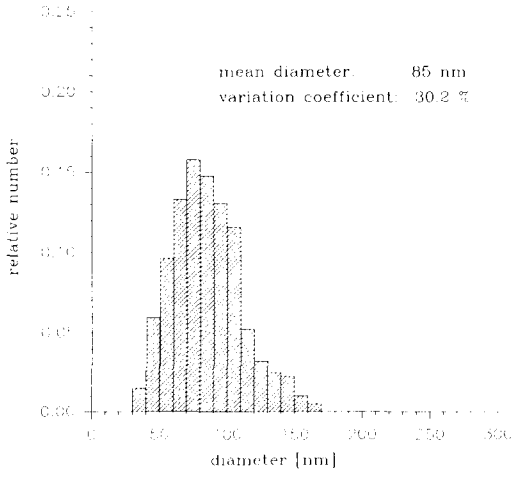

Fig. 7. Number weighted vesicle size distribution of Gd-DTPA-carrying liposomes determined by negative staining TEM. Lipid composition: SPC:Chol (7:3). Lipid concentration, 150 mg/g; Gd concentration, 180 μmol/g; three freeze-thaw cycles; final pore size, 0.1 μm.

for which a gaussian analysis fit has been used and unimodal distributions have been obtained.

4. Discussion

Extrusion of contrast-carrying liposomes with the new continuous high pressure device was fast and easy. By application of polycarbonate membranes with smaller pore sizes during the final extrusion step, we could reduce the mean diameter of liposomal preparations. At the same time, the encapsulation efficiency also decreased, however, but not to an extent that would be expected because of the sharp decline in vesicle size. The diminution of the mean diameter from 210 to 90 nm only caused a reduction of iopromide entrapment from 50 to 34%. For Gd-DTPA the influence of size reduction on encapsulation was even smaller. The results indicate that besides the mean diameter, lamellarity is also affected by employment of small pore sizes (Hope et al., 1985; Mayer et al., 1985). Although the encapsulated volume of a single vesicle is reduced with decreasing diameter, the formation of several smaller unilamellar vesicles out of one large multi- or oligolamellar vesicle during the process of extrusion will restrict the overall reduction of encapsulation efficiency. For our purpose vesicle diameters of about 100 nm, which were yielded with a final pore size of 0.1 μm, have been sought.

As expected, the entrapment of contrast agents could be improved by increasing the lipid concentration, however, at the cost of declining contrast agent to lipid ratio. Since differences in vesicle size could not be detected by PCS, we believe that the decline, which has also been described for other liposome systems (Benita et al., 1984; Seltzer et al., 1988), might be the result of a growing lamellarity within the vesicular population at higher lipid concentrations.

The effect of increasing contrast agent concentration was quite opposite to that described for lipid concentration. Encapsulation efficiencies fell sharply when higher solute concentrations were used. For radiographic contrast agents, this phenomenon has also been described by others (Ryan

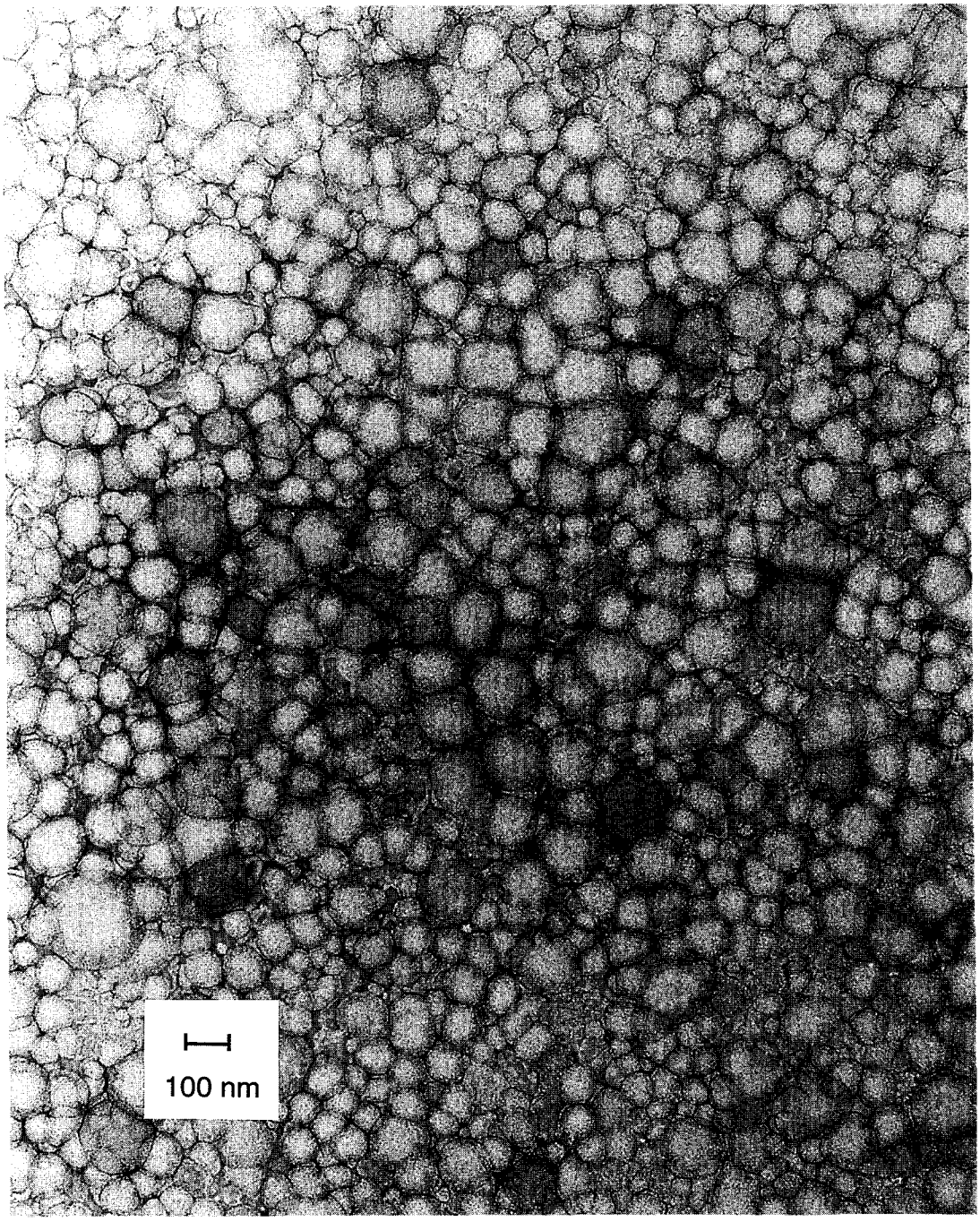

Fig. 8. Negative staining electron micrograph of continuously extruded Gd-DTPA-carrying liposomes. Lipid composition: SPC : Chol (7 : 3). Lipid concentration, 150 mg/g; Gd concentration, 180 μmol/g; three freeze-thaw cycles; final pore size, 0.1 μm.

et al., 1983; Benita et al., 1984; Cheng et al., 1987; Seltzer et al., 1988), however, in this work we were able to show it for the MRT contrast agent Gd-DTPA as well. The authors of previous papers believe that vesicle formation is somehow disturbed at high solute concentrations either due to a high viscosity or because of increasing osmotic pressure.

High encapsulation efficiencies were achieved, when a freeze-thaw protocol was employed. A reason for the observed improvement of liposomal entrapment might be that small vesicles, which are destroyed during freezing will fuse to form vesicles with increasing diameters during thawing, thus encapsulating larger volumes of the aqueous phase (Hope et al., 1985).

Experiments carried out with various lipid compositions revealed interesting differences regarding encapsulation efficiency and stability between the two contrast agents. Whenever the non-ionic iopromide was used, the encapsulation could be improved by addition of negatively charged lipids (SPG or DCP) to a mixture of SPC and Chol. Furthermore, negative charge prevented aggregation and subsequent fusion, and thus improved the stability of iopromide-carrying liposomes. Others, who have described the effect of increasing entrapment for sonicated liposomes, suggested that it might be caused by a decrease of lamellarity and an increase in interlamellar distance (Benita et al., 1984) due to electric repulsion forces between bilayers. Whether this theory represents a possible explanation for our results remains uncertain, since data about lamellarity of continuously extruded vesicles have not been obtained yet.

In vesicular systems containing the negatively charged Gd-DTPA molecule as well as SPG or DCP, repulsive forces, this time arising between bilayers and solute, caused instability and, in addition, prevented any improvement in encapsulation efficiency.

The reason why CholHS, which is a negatively charged lipid as well, had surprisingly no effect on the encapsulation neither a positive for iopromide, nor a negative for Gd-DTPA might be that, in contrast to the carbohydrate chains of SPG or DCP, the sterol of CholHS is incorporated into the membrane in a way that leads to a shielding of the negative charge and prevents its exposure on the bilayer surface. Zeta potential determination in this work showed smaller values for CholHS-containing liposomes compared to SPG or DCP, thus possibly supporting the above-mentioned hypothesis.

Long-term stability of liposomal preparations made with optimum lipid compositions (SPC:Chol:SPG 6:3:1 for iopromide and SPC:Chol 7:3 for Gd-DTPA) was satisfactory. Storage at 2–8°C did not affect macroscopic appearance and amount of entrapped solutes. There was only a slight increase in mean diameter, which is unlikely to influence the biodistribution of stored liposomes. We propose that especially the fact that unencapsulated solute was not removed from the preparation plays an important role in stabilizing the vesicle suspension. Regarding the high encapsulation efficiencies achieved here, removal of the unentrapped material before diagnostic application might not be necessary, especially since the compounds employed are highly tolerable (Sachse et al., 1993).

TEM results indicated that the continuous high pressure extrusion method yielded unimodal vesicle populations with small size distributions. Considering that TEM with different visualization methods, such as negative staining and freeze fracture, already leads to deviating results regarding vesicle diameters (Olson et al., 1979), our PCS data are in good agreement with those obtained from TEM.

The results discussed so far prove that the new continuous high pressure extrusion method in combination with a freeze-thaw protocol is suitable for the fast and simple production of large batches of stable, contrast-carrying liposomes with surprisingly high encapsulation efficiencies. Employing optimum production parameters, we obtained entrapment values of more than 50% for the water-soluble, nonionic X-ray contrast agent iopromide and over 60% for the ionic MRT contrast agent Gd-DTPA. The results exceed the highest values published so far for passive entrapment of water-soluble X-ray and MRT contrast agents (Tilcock et al., 1989; Sachse et al., 1993). These outstanding results could be achieved al-

though the mean vesicle diameters in the preparations, as determined by PCS, were only about 100 nm.

For reasons of economy we used volumes between 100 and 200 ml for extrusion in this work, although we have already produced continuously extruded liposomes in 1 l batches (data not shown). These volumes are well above the maximum batch sizes reported so far for most discontinuous extrusion methods (Szoka et al., 1980; Hope et al., 1985; Nayar et al., 1989; Unger et al., 1991). Due to the continuous process and high pressures that lead to filtration flows of up to 500 ml/min, rapid extrusion of large batch volumes has now become possible. Even a further upscaling of the new method to an industrial scale should be easy to accomplish. Thus, a major breakthrough has been achieved with the introduction of the continuous extrusion process.

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