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International Journal of Pharmaceutics 128 (1996) 89–98

international journal of pharmaceutics

Preparation and stability of interferon-α-containing liposomes

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Received 1 May 1995; accepted 9 June 1995

Abstract

Several liposome types containing human interferon-α (hIFN-α) were produced and were assessed to determine the levels of hIFN-α activity associated with surface-adsorbed or interior-entrapped hIFN-α. Liposomes were prepared as multilamellar vesicles (MLV) following repeated extrusion through 0.2 μm polycarbonate filters, and by a mechanical high shear homogenization system using the one-step method. The highest trapping efficiency for hIFN-α, at around 23%, was found for liposomes composed of egg phosphatidylcholine/cholesterol/dimyristoyl phosphatidylglycerol in a 6:4:1 molar ratio, prepared at a lipid content of 10% (w/v) by extrusion. The trapping efficiency for hIFN-α in homogenized liposomes was only around 3%. hIFN-α liposomes prepared by extrusion were stable over 4 months at 20°C, with regard to the activity of hIFN-α and the size distribution of the liposomes.

Keywords: Extrusion; Interferon-α; Liposomes; Microfluidization; Particle size; Stability

1. Introduction

Leukocyte-derived interferon-α (IFN-α) is a naturally occurring protein, and has antiviral, antiproliferative and some immunomodulatory effects (Pestka et al., 1985; Gastl and Huber, 1988; Gresser, 1990; Knop, 1990; Baron and Dianzani, 1994). Because of these properties IFN-α has found its place in therapy of several viral diseases, and exhibits encouraging anticancer activities (Steinmann et al., 1994). Liposomes as drug delivery systems have the potential for providing controlled release of the administered drug and increasing the stability of labile drugs (Gregoriadis, 1991), such as IFN-α. Liposomal IFN-α preparations can alter the pharmacokinetics, tissue distribution and uptake of IFN-α in comparison with free IFN-α (Eppstein and Stewart, 1982; Eppstein, 1982). In cancer therapy, liposomal IFN-α can improve therapeutic results by obviating a rapid dilution and/or degradation of interferon in the organism, and also increase the convenience of therapy by allowing less frequent drug administration (Killion et al., 1989). The mode of association between IFN-α and phospholipid vesicles, i.e. surface-adsorption or entrapment, may have an influence on these findings (Anderson et al., 1981; Eppstein and Stewart, 1981).

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SSDI 0378-5173(95)04227-2

A variety of techniques have been described for preparing liposomes, including drug-containing liposomes (Batzri and Korn, 1973; Szoka and Papahadjopoulos, 1978; Szoka and Papahadjopoulos, 1980, Shew and Deamer, 1985; Martin, 1990). Most of them provide heterodisperse suspensions in which the liposomes size distribution can be reduced by a number of known size-processing methods, e.g. sonication (Johnson et al., 1971) or extrusion of MLV through polycarbonate membranes with defined pore sizes (Olson et al., 1979). The pro-liposome method (Williams, 1992), the 'bubble' method (Talsma et al., 1994), detergent-induced liposome loading (Schubert, 1990) or homogenization (Mayhew et al., 1984; Mayhew et al., 1987) are one size-processing methods suitable for entrapment of hydrophilic molecules and for large-scale production.

Liposomal IFN-α has generally been prepared by the film method, by sonication of MLV or by reverse phase evaporation (Eppstein and Stewart, 1982). Stability of liposomal IFN-α has been tested for at least 1 month (Eppstein and Stewart, 1981).

The purpose of this study was to investigate the effect of the composition and preparation method of liposomes on the levels of activity of hIFN-α, dispersed as free hIFN-α in the liposome suspension, adsorbed at the surface of the phospholipid bilayers and entrapped in the aqueous compartments of the liposomes. The stability of negatively charged liposomes with the highest trapping efficiency for hIFN-α is reported, with regard to the activity of hIFN-α and the size distribution of the liposomes.

2. Materials and methods

2.1. Materials

HIFN-α, polyclonal antibodies against hIFN-α (PAB) and murine monoclonal antibodies (MAB) were produced by BioNative AB, Umeå, Sweden. Egg yolk phosphatidylcholine (PC) (Type Lipoid E 100), phosphatidylglycerol sodium (PG) (Type Lipoid PG), and dimyristoyl phosphatidylglycerol sodium (DMPG) (Type Lipoid PG 14:0/14:0) were obtained from Lipoid KG, Ludwigshafen, Germany. Cholesterol 96% (Chol), *dl*-α-tocopherol, human serum albumin (HSA), alkaline phosphatase-conjugated anti-mouse IgG and *p*-nitrophenyl phosphate disodium were from Sigma, Deisenhofen, Germany; immunoplates (96-well) from NUNC, Wiesbaden, Germany; Sepharose CL-4B from Pharmacia LKB GmbH, Freiburg, Germany; and Tween 20 from ICI Surfactants, Essen, Germany. Organic solvents were freshly redistilled before use, and cholesterol was recrystallized three times from methanol. All other reagents were of analytical grade.

2.2. Methods

2.2.1. Liposome preparation by extrusion

Each mixture of 1.0 g lipids (PC/Chol in a 6:4 molar ratio at lipid contents of 2%, 5% and 10%; PC/Chol/PG in a 6:4:1 molar ratio at a lipid content of 10%; PC/Chol/DMPG in a 6:4:1 molar ratio at a lipid content of 10%) containing 0.1% *dl*-α-tocopherol was dissolved in about 70 ml chloroform, and the volume adjusted to 100 ml with chloroform. An aliquot of the lipid solution was then transferred to a round-bottomed flask following evaporation under a stream of nitrogen. The resulting dry lipid film was held under a vacuum of 0.17 bar at 30°C for 60 min to remove any traces of solvent. It was then hydrated at the specified lipid concentration with TRIS buffer [pH 7.4, containing 1.5 mg/ml HSA and 0.02% NaN_3 (w/v)] containing 600 000 U/ml hIFN-α by rotation of the flask which contained small glass beads. The resulting suspension, containing MLV, was subjected to 21 passes through 200 nm polycarbonate filters (Costar, Bodenheim/Mainz, Germany) mounted in a mini-extruder (type LiposoFast, Avestin Inc., Laudenbach, Germany). An odd number of extrusions was chosen to avoid contamination of the final liposome suspension with large vesicles which might have not passed the polycarbonate membrane. Oligolamellar vesicles with a narrow size distribution were obtained.

2.2.2. Liposome preparation by homogenization

The mixture of 2.0 g lipids (PC, 1.75 mmol; Chol, 1.17 mmol; DMPG, 0.29 mmol; and *dl*-α-tocopherol, 2.0 mg) was suspended in 20 ml TRIS buffer containing 600 000 U/ml hIFN-α and stirred for 1 h at room temperature. For homogenization, a small volume microfluidizer for sample volumes from 12.5 ml to 25 ml (model M-110S, Microfluidics Corp., Newton, Massachusetts, USA) with compressed air and a cooling coil in ice water was used. The raw lipid suspension was added to the valve reservoir and the pump was run slowly until air entrained in the system was accumulated in the valve reservoir. The pump was then re-started and the suspension was pumped under high pressure through the interaction chamber of the microfluidizer, until the desired average size of liposomes was achieved. The temperature of the liposome suspension in the valve reservoir was 16°C, and the air pressure was 6 bar, corresponding to 840 bar in the interaction chamber. For measuring the particle size distribution by laser diffraction the pump was shut off every 10 min and a sample of 1 ml was drawn.

2.2.3. Size measurements of liposomes by dynamic laser light scattering

The liposome suspension (10% lipid concentration) was diluted around 3000 times in TRIS buffer. Dust particles in the sample were eliminated by filtering the diluted liposome suspension through a sterile 800 nm pore size filter. The diameter of the liposomes was estimated by photon correlation spectroscopy (PCS), using a submicron particle analyzer (Coulter, model N4S, Coulter Electronics GmbH, Krefeld, Germany) at 25°C, with a scattering angle of 90.0 degrees, a refractive index of 1.333, a sampling time of 30.0 μs and a run time of 200 s. Liposome concentrations measured were in the range of 10^4– 10^5 counts/s. The data were analyzed using the SDP program according to CONTIN (Provencher, 1982).

2.2.4. Size measurements of liposomes by laser-diffraction

Measurements were performed at 22–23°C, using a Helos Submicron particle-size analyzer (Sympatec GmbH, Clausthal-Zellerfeld, Germany) with a suspension closed-loop cell (type SUCELL 12CL) and Helos- and QX-software. The dilution medium was distilled water. Liposome suspension was added until an optical density of 4–10% was obtained. A lens with a focal distance of 20 mm (measuring range 0.1–35 μm), sonication time of 60 s with a break of 5 s before measurement and measurement time of 5 s were chosen for all measurements.

2.2.5. Purification of liposomes

Free and liposomal (adsorbed and entrapped) hIFN-α were separated by gel filtration. First, 100 μl of the liposome suspension were filtered at 10°C using a jacketed Sepharose CL-4B column of 1.0 cm diameter, a gel bed height of 18 cm, and TRIS buffer was used as the medium. A flow adaptor was adjusted to the column and fractions of 1.46 ml were collected at a flow rate of 12.1 ml/h.

2.2.6. Association of hIFN-α with liposomes

After purification the modes of association of hIFN-α with liposomes were assessed by ELISA (described in Section 2.2.9.), in the following samples. Firstly, fractions containing free hIFN-α were assessed without any further treatment. Secondly, fractions containing both adsorbed and entrapped hIFN-α were assayed without any further treatment. This represents the hIFN-α adsorbed at the surface of the liposomes. Thirdly, aliquots of these fractions were incubated for 1 h at 8°C with Triton X-100, at a final detergent concentration of 1.8 mg/ml. The total of recovered liposome hIFN-α [adsorbed and entrapped (released)] was then determined. The amount of hIFN-α released from liposomes after solubilization is expressed as the calculated difference between the total recovered hIFN-α and the adsorbed hIFN-α.

2.2.7. Stability studies

HIFN-α liposomes (molar ratio of PC/CH/DMPG, 6:4:1) with a lipid concentration of 10%, prepared by repeated extrusion, were stored in an incubator at 20°C for 125 days and 37°C for 74 days, respectively. The samples were then filtered

and assayed for hIFN-α, as described in Section 2.2.5. and Section 2.2.6.

2.2.8. Cryo-transmission electron microscopy (cryo-TEM)

Liposome samples were diluted with TRIS buffer to a lipid concentration of around 1%. A drop of the sample was placed on the surface of a porous glow discharged copper grid held by tweezers and mounted on the spring-loaded plunger of the Controlled Environment Vitrification System (CEVS). The CEVS was equilibrated with liquid nitrogen. Excess sample was removed by touching a filter paper to the grid. The specimen was plunged by a spring-driven device through the opening shutter and into the cryogen container filled with liquid ethane at its freezing point, where the thin specimen films were vitrified. Mounted on a precooled cryo holder with two metal shields, the vitreous specimen was transferred into a TEM container filled with liquid nitrogen, and into the TEM, where the specimen was maintained at 90 K. The specimen was imaged using low-dose techniques. Specimens were examined in an electron microscope (model ZEISS CEM 902) with an EM-Specimen Cooling Holder. Micrographs were recorded on Scientia film (Agfa).

2.2.9. Enzyme linked immunoassay (ELISA)

Immunoplates were coated with an isotone phosphate buffer (PBS) containing the PAB (4 μg/ml) for 2 h at 37°C. Unspecific binding sites were blocked by treating the plates with HSA, 5 mg/ml in PBS for 2 h at 37°C. Samples were serially diluted with TRIS buffer. The diluted samples and the MAB (5 μg/ml in TRIS buffer) were incubated for 12 h at room temperature. Bound molecules were detected using alkaline phosphatase-conjugated anti-mouse IgG solution (1:2000 dilution in TRIS buffer) by incubation for 2 h at room temperature. Between the incubation steps, the plates were washed three times with Tween 20 solution [0.05% (w/v)]. The reaction was visualized using a diethanolamine buffer (pH 9.8), containing *p*-nitrophenyl phosphate (1 mg/ml) as enzyme substrate. The resulting colour reaction was measured at 405 nm using an ELISA spectrophotometer (Labsystems, Helsinki, Finland). When the absorbance of the highest standard sample exceeded 1.0, measurements were stopped and 1 M NaOH was added in order to stop the enzyme reaction until further readings were taken. The data were analyzed by computer package (SeroCalc, Bornheim-Hersel, Germany). Each assay included a standard curve of hIFN-α in the range 0–6000 U/ml.

3. Results

3.1. Influence of lipid composition on the trapping efficiency of liposomes

The levels of hIFN-α activity due to adsorbed and to entrapped hIFN-α prepared by repeated extrusion are given in Fig. 1. Neutral liposomes (molar ratio of PC/Chol, 6:4) showed large levels of hIFN-α activity due to adsorbed hIFN-α. The activity increased from 5.7 ± 1.3% to 55.8 ± 14.5% when the lipid concentration was increased from 2 to 10%. The low activity of entrapped hIFN-α was only around 4% at a lipid concentration of 10%.

The addition of either negatively charged lipid components, PG or DMPG, caused both an in-

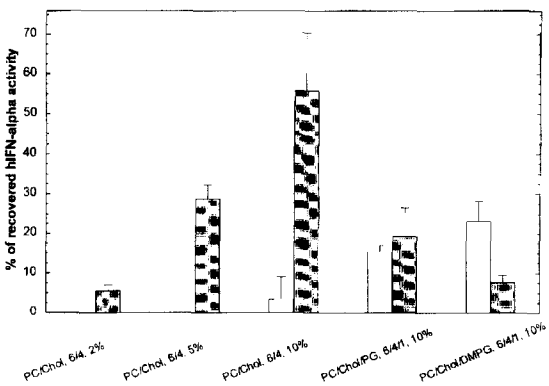

Fig. 1. % of recovered hIFN-α activity from hIFN-α liposomes. Liposomes of different lipid compositions and lipid concentrations as indicated were prepared by multiple extrusion through polycarbonate membranes and separated from free hIFN-α by gel filtration. The white bars represent the recovered activity of entrapped hIFN-α, and the dark bars represent the activity of hIFN-α adsorbed at the surface of the liposomes.

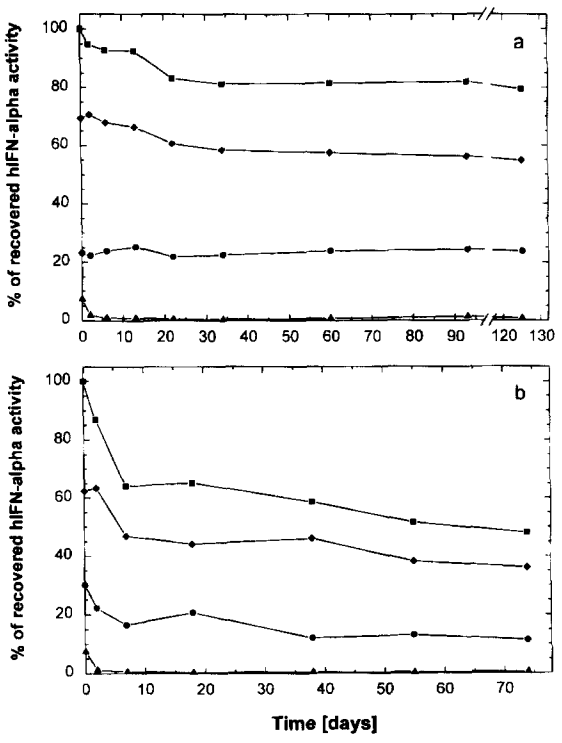

Fig. 2. % of recovered hIFN-α activity from hIFN-α liposomes (molar ratio PC/Chol/DMPG, 6:4:1) prepared by repeated extrusion and stored at 20 °C (a) and 37 °C (b), respectively. Samples of hIFN-α liposomes were separated after indicated times by gel filtration. The fractions obtained were assayed for free hIFN-α (◆), hIFN-α entrapped in liposomes (●) and hIFN-α adsorbed at the surface of liposomes (▲). The sum of free, adsorbed and entrapped hIFN-α is also shown (■).

crease in the activity due to entrapped hIFN-α and a decrease in the activity due to adsorbed hIFN-α. The highest trapping efficiency was obtained by incorporating dimyristoyl phosphatidylglycerol (molar ratio of PC/Chol/DMPG, 6:4:1) with levels of entrapped hIFN-α of 23.2 ± 5.0%. The incorporation of phosphatidylglycerol (molar ratio of PC/Chol/PG, 6:4:1) produced levels of entrapped hIFN-α of 15 ± 1%.

3.2. Stability of liposomal hIFN-α at 20°C and 37°C

Preliminary stability studies at 8°C had indicated that liposomal hIFN-α was stable for at least 3 months (data not shown). At a storage temperature of 20°C (Fig. 2a) there was a loss of around 17% from the initial total activity of recovered hIFN-α after 22 days. During the following 103 days the total activity remained constant. Only the free hIFN-α in the liposome suspension showed a decrease in activity of 6% of the initial value after 22 days. The activity of entrapped hIFN-α was stable over the whole storage time, and the activity of adsorbed hIFN-α decreased approximately to zero within 6 days. At a storage temperature of 37°C (Fig. 2b) a loss of 36% of the initial total activity of recovered hIFN-α was found within 7 days. During the following 67 days the total activity of recovered hIFN-α decreased continuously to 48% of the initial value. Similarly, the activity of free hIFN-α and also the activity of entrapped hIFN-α decreased within the first 7 days of storage. The activity of adsorbed hIFN-α decreased to zero within 2 days.

3.3. Size distribution and shape of hIFN-α liposomes prepared by repeated extrusion

Multilamellar vesicles (100 mg/ml total lipid) containing 600 000 U/ml hIFN-α at a lipid molar ratio PC/Chol/DMPG, 6:4:1 were subjected to increasing numbers of extrusion. After seven extrusion steps the liposome suspension was still somewhat cloudy, the size of the vesicles being around 200 nm (Table 1). A further reduction in size was achieved by increasing the number of extrusion steps from seven to 15. A homogeneous opalescent dispersion was obtained with liposomes sized around 185 nm. Liposome extrusion with 21 steps yielded liposomes having an average size of 176 ± 21 nm (five independent liposome preparations), and a narrower size distribution than that obtained after 15 steps. Cryo-TEM revealed the liposomes obtained after 21 extrusion steps to be spherical shells with an average of two lamellae (Fig. 3). All three liposome preparations showed high stability during 4 months at 8°C, with regard to size and size distribution.

3.4. Size distribution and trapping efficiency of hIFN-α liposomes prepared by homogenization

During the first 30 min of homogenization broad, bimodal and variable liposome size distri-

Table 1
Liposome size distributions of hIFN-α liposomes determined by photon correlation spectroscopy after indicated times at 8°C. Multilamellar vesicles (100 mg/ml total phospholipid, 600 000 U/ml hIFN-α) were prepared by the thin lipid film procedure at a lipid ratio PC/Chol/DMPG, 6:4:1 and extruded through one polycarbonate membrane of 200 nm pore size

Number of extrustion steps	Storage period (days)	Intensity Results		
		Mean size(nm)	S.D.(nm)	C.V.(%)
7	1	197	35	18
	8	194	36	19
	27	195	34	17
	80	201	48	24
	127	198	36	18
15	1	185	30	16
	8	202	48	24
	27	179	26	14
	80	183	20	11
	127	185	50	25
21	1	176	23	13
	8	191	29	15
	27	177	24	14
	80	183	20	11
	127	173	17	19

S.D., standard deviation.
C.V., coefficient of variation.

butions with distribution peaks between 0.53 to 0.79 μm depending on the homogenization time were obtained (Fig. 4a,b). During the following 50 min of recirculation, further reductions in size were obtained with the distribution peak diameters being 0.45 μm after 40 min and 0.26 μm after 80 min. The trapping efficiency measured after a homogenization time of 80 min was 2.7%, and the levels of adsorbed hIFN-α activity were 4.4% (Table 2). However, the activity of free hIFN-α decreased from 40.2% after 60 min of homogenization to 34.6% after 80 min of homogenization.

4. Discussion

The extent of entrapment of water-soluble molecules in liposomes is influenced by the lipid composition and concentration, the size of the vesicles, and the preparation technique. Extrusion of MLV and microfluidization were the techniques chosen for the preparation of hIFN-α liposomes. Both techniques satisfy pharmaceutical requirements for liposomes of uniform size distributions and long term stability.

The size distribution of vesicles obtained by gentle hydration of a dry lipid film followed by swelling in an excess of water is very broad. Also, it is difficult to obtain reproducible MLV preparations, even with standardized procedures. Liposomes of size below around 0.4 μm show lower tendency to agglomerate on storage than larger-sized ones. However, liposomes of a size of less than 0.08 μm, have limited drug capacity. Therefore, polycarbonate membranes with a pore diameter of 200 nm were chosen to prepare liposomes using a small-volume extrusion apparatus. This apparatus allows processing volumes of up to 1 ml, in the preparation of large, unilamellar vesicles (MacDonald et al., 1991). As shown in Fig. 2a, hIFN-α can be associated with liposomes in different ways, mainly by being trapped in the aqueous interstices or by being adsorbed at the surface of liposomes. The predominant mode of association between interferon and liposomes has been identified as passive entrapment (Anderson et al., 1981). Hence a mol-percentage of 36% cholesterol (Fig. 1) was chosen to minimize liposome permeability for the entrapped hIFN-α. A stable entrapment of hIFN-α for more than 4

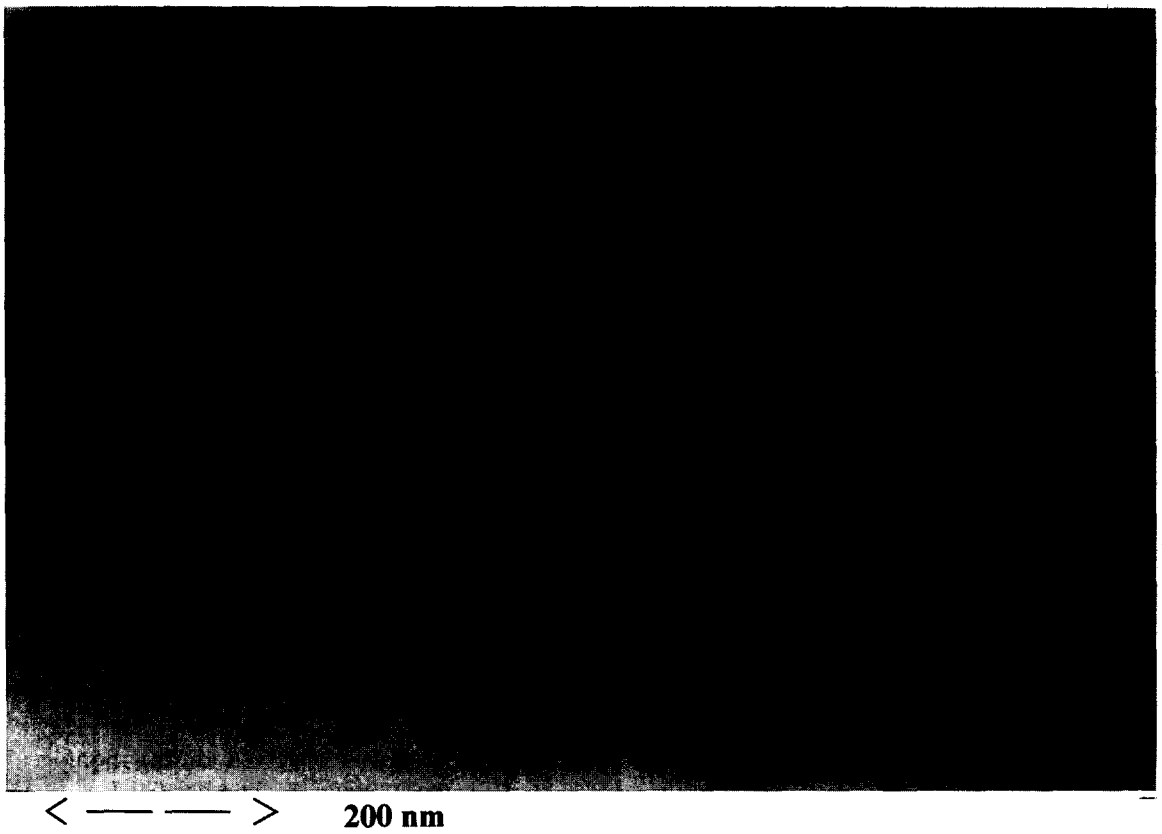

Fig. 3. Cryo-electron micrograph of unstained hIFN-α liposomes after cryofixation. Liposomes were prepared by repeated extrusion of multilamellar liposomes through polycarbonate membranes with a defined pore size of 0.2 μm and were stored at 8°C for 2 days.

months at 20°C was obtained using negatively charged liposomes (PC/Chol/DMPG, 6:4:1), prepared by repeated extrusion, with a lipid concentration of 10% (Figs. 1 and 2a). The presence of a negatively charged component is necessary for a stable association, and the incorporation of IFN-α into liposomes is even better when cholesterol is included in the lipid bilayer (Eppstein and Stewart, 1982). The initial loss in total activity appears to have been mainly caused by a loss of free hIFN-α since the activity of entrapped hIFN-α was stable over the whole storage time, and the activity of adsorbed hIFN-α decreased approximately to zero within 6 days (Fig. 2a). Incubation of the extruded liposomes containing hIFN-α at 37°C caused a marked leakage of entrapped hIFN-α after 7 days and an initial decrease in the levels of activity due to adsorbed hIFN-α within two days (Fig. 2b). These results are in accordance with those previously reported (Eppstein and Stewart, 1981).

The stability of liposomes can be characterized and controlled with regard to their shape, and size distribution by various methods such as light microscopy, electron microscopy, dynamic light scattering techniques, analytical ultracentrifugation, NMR spectroscopy and gel chromatography. HIFN-α liposomes prepared by extrusion were characterized by PCS and cryo-transmission electron microscopy, respectively. These liposomes being relatively homogeneous in size and structure were found to be stable over 4 months at 8°C (Table 1). Dynamic laser light scattering is a suitable method for determining size distributions of liposomes consisting of a rather homogeneous population with well-defined shape (Ruf et

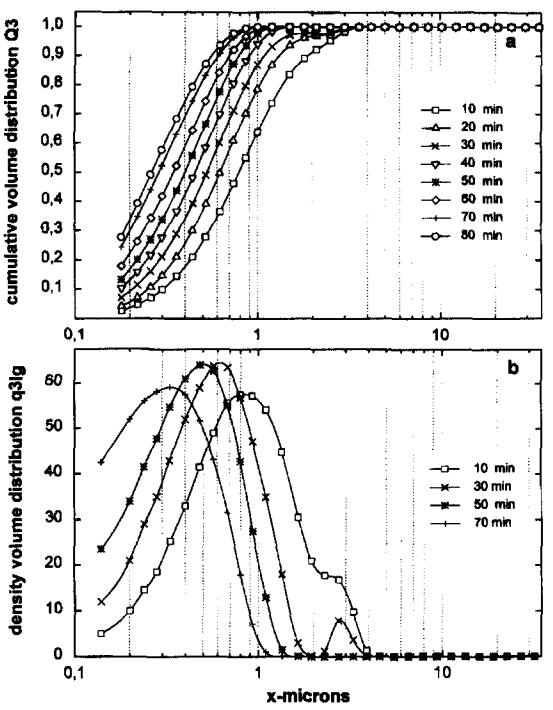

Fig. 4. Cumulative (a) and density (b) volume distributions of hIFN-α liposomes prepared by homogenization using a microfluidizer and analyzed by laser diffraction after indicated times of homogenization at an air pressure of 6 bar.

al., 1989; Ostrowsky, 1993). Contrary to earlier findings (MacDonald et al., 1991), cryo-TEM images revealed that the liposomes were not unilamellar, but mostly consisted of two lamellae (Fig. 3). Although electron microscopy has the disadvantage that liposomes have to be treated additionally, which may affect their structure and size distribution, it is a convenient technique to assess the shape and lamellarity of liposomes. The results obtained suggest that the shape of the liposomes is not only dependent on the method of preparation but also on the lipid composition.

For comparison purposes an attempt was made to prepare hIFN-α liposomes by homogenization using a microfluidizer according to the one-step method. As a lab-scale liposome preparation technique, this system offers the advantages that large amounts of small-sized liposomes can be produced continuously and that it is more efficient at high lipid concentrations, thus providing high trapping efficiencies of water-soluble agents. High yields for the entrapment of aqueous soluble molecules in liposomes have been already achieved by homogenizing preformed liposomes (Mayhew et al., 1984), and by the one-step method (Bauer, 1992). In the present study, while the trapping efficiency for hIFN-α obtained is low (approximately 2.5%), the activity of adsorbed hIFN-α is higher than in liposomes prepared by extrusion (Table 2). A marked loss of activity of free hIFN-α was observed during homogenization. In the interaction chamber fixed microchannels provide an extremely focused interaction zone. The liposome suspension is accelerated to a high velocity and subjected to intense shear, impact, and cavitation. However, the resulting liposome size distribution was quite broad and variable, depending on a number of process variables, such as pressure, homogenization time and internal temperature. Analyzing size distributions of these liposomes by PCS resulted in a bimodal

Table 2
Percentage of recovered hIFN-α activity from liposomes (molar ratio PC/Chol/DMPG, 6:4:1) with a lipid concentration of 100 mg/ml

Preparation method	Total recovered activity (% of initial activity)	Free hIFN-α (% of initial activity)	Adsorbed hIFN-α (% of initial activity)	Entrapped hIFN-α (% of initial activity)	Efficiency[c] (%)
Extrusion[a]	77.7	59.9	1.5	18.3	23.6
Homogenization[b]					
for 60 min	45.7	40.2	4.4	1.1	2.4
for 80 min	40.5	34.6	4.9	1.1	2.7

[a]21 passes of a MLV containing liposome suspension through 200 nm polycarbonate membranes.
[b]Homogenization according to the one-step method at an air pressure of 6 bar.
[c]Efficiency: trapping efficiency.

distribution possibly due to the presence of large vesicles which upset the measurement. Therefore, laser diffraction was preferred. Liposome size and size distribution became smaller by increasing the number of cycles in the interaction chamber (Fig. 4).

These results show that trapping efficiencies of 23 ± 3% for hIFN-α can be easily obtained preparing liposomes by extrusion, using negatively charged liposomes and high lipid concentrations. Further investigations are required to establish suitable experimental conditions for preparation of liposomes by homogenization.

Acknowledgements

This work was kindly supported by Dr. Rentschler Arzneimittel GmbH and Co. (Laupheim, Germany). Our thanks are also due to Lipoid KG (Ludwigshafen, Germany) for their generous gifts of lipids and to Microfluidics Corp. (Newton, MA, USA) for placing the microfluidizer at our disposal. The authors are grateful to Margret Schmidtgen and Markus Drechsler for their excellent cryo-TEM micrographs.

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