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CHARACTERIZATION OF LIPOSOMES PREPARED USING A MICROEMULSIFIER

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A new type of device can prepare liposomes continuously, in large quantities and with excellent aqueous space capture efficiency. At initial lipid concentration of 300 μmol/ml these liposomes capture approx. 75% of cytosine arabinoside used as an aqueous space marker. Liposome size can be reduced by increasing the number of times the preparations are recycled through the microemulsifier. Liposomes less than 0.1 μm in diameter, as shown by electron microscopy, can be made easily. Liposomes prepared at 300 μmol/ml, composed of phosphatidylglycerol/phosphatidylcholine/cholesterol in a 0.1:0.4:0.5 molar ratio leaked less than 1% of entrapped cytosine arabinoside (Ara-C) at 4°C, and less than 10% Ara-C at 37°C plus serum, over a 48 h period. These liposomes could be useful for a number of applications including diagnostics, therapeutics and model membrane studies.

Introduction

Liposomes have been exploited in the laboratory for diverse research, diagnostic and pharmacologic applications. Practical extension of this work has been limited by lack of a technology that might allow the reproducible manufacture of liposomes of predictable, uniform size distribution. A number of different methods have been described for preparing liposomes [1,2] which, in general, tend to be cumbersome, poorly reproducible, or limited in production batch size. These problems appear to have been overcome by a new microemulsification technique described here. Using principles of fluid dynamics, this device produces liposomes by a continuous process in a manner compatible with pharmaceutical Good Manufacturing Practices. The process is reproducible and yields liposomes of well-defined size distributions, with good aqueous phase encapsulation efficiency. Liposomes can be prepared by this method in amounts sufficient for pharmaceutical quality assessment, toxicology studies and multicenter clinical trials.

Materials and Methods

Lipids and cholesterol were prepared, purified and stored as described previously [3]. The lipids used in the described studies were phosphatidylglycerol (PG) (Calbiochem-Behring, San Diego, CA), egg phosphatidylcholine (PC), and cholesterol in the molar ratio of (PG/PC/Chol) 0.1 : 0.4 : 0.5. The lipids in chloroform were dried extensively under reduced pressure. Aqueous solvent containing [^{3}H]cytosine arabinoside was used as it is entrapped in the aqueous space in liposomes and

Abbreviations: Ara-C, cytosine arabinoside; PG, phosphatidylglycerol; PC, phosphatidylcholine.

has been used extensively in chemotherapeutic studies with liposomes [3,4]. The hydrated lipid was then processed by the microemulsifier (see Fig. 1).

The microemulsifier (Microfluidizer™ M110, patent pending) was invented at Arthur D. Little Inc. (Boston, MA) and developed by Biotechnology Development Corporation (BDC, Newton, MA). Fig. 1 shows a schematic of the M110. Hydrated lipid suspension is added to the Reservoir and enters the pneumatic Pump (the air stream and fluid streams do not come into contact thus eliminating the necessity of using an inert gas). The fluid is pumped under high pressure (10000 lb/inch^2) through a filter (5 μm) into the Interaction Chamber where it is separated into two streams which interact at extremely high velocities in dimensionally defined microchannels. The suspension can be used after a single pass or recycled through the Reservoir. Typically, volumes of 15 ml and a flow rate of 90 ml/min were used. In the experiments described in this report the main variables investigated were recycling time and initial lipid concentrations.

After preparation, the liposomes were separated from non-entrapped drug by exhaustive dialysis. Samples were then taken for determination of permeability and drug capture as described previously [3,5] using multiple changes of dialysis bags.

Electron microscopy was carried out by suspending the liposomes in 30% glycerol. The suspension was then frozen in Freon 22 and fractured at $2 \cdot 10^{-6}$ torr vacuum. Replicas were floated on water and transferred to 5% sodium hypochlorite

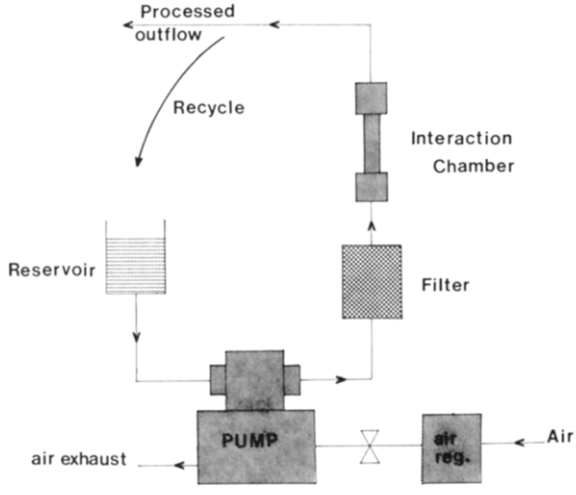


Fig. 1. Scheme of the Microfluidizer™ M110.

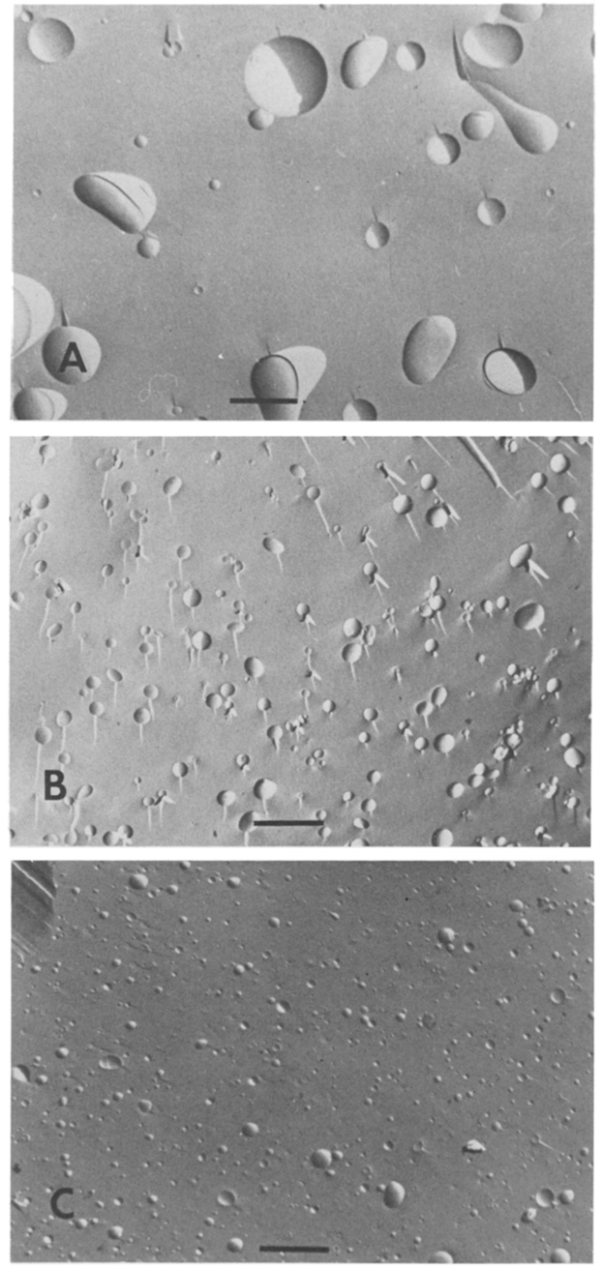


Fig. 2. Freeze-fracture electron microscope photos of liposomes. (A) Hydrated lipid (crude multilamellar vesicles) before microemulsification. (B) Microemulsified liposome preparation after 2 min recycling using the Microfluidizer™. (C) Microemulsified liposome preparation after 10 min recycling using the Microfluidizer™. (Bar represents 1.0 μm in all photos.)

and washed in water. The replicas were mounted on copper grids and examined using a Siemens 1A Elmskop [5].

Sonicated unilamellar liposomes, multilamellar liposomes and reverse phase evaporation (mainly large and unilamellar) liposomes were prepared, using Ara-C as the aqueous space marker, as described previously [2,5]. Extrusion of multilamellar vesicles and reverse phase evaporation liposomes was carried out using nucleopore membranes to produce smaller liposomes of a more homogeneous size distribution [5–7]. These preparations were compared with liposomes formulated using the Microfluidizer™ M110 which did not require a membrane extrusion step.

Results

Fig. 2A shows the appearance of hydrated lipids before microemulsification. These liposomes are similar in size to a normal hand-shaken multilamellar liposome preparation before extrusion [6]. The size of the liposomes is in the +1 μm range and very few smaller liposomes are visible. Fig. 2B shows the appearance of the preparation after 2 min microemulsification. The size is reduced to an approximate mean diameter of about 0.1–0.2 μm. It can be seen that the size distribution is quite narrow. Fig. 2C shows the appearance of the preparation after 10 min microemulsification. The size of most of the Microfluidizer™ liposomes is further reduced to less than 0.1 μm. Increasing the emulsification time beyond 10–20 min did not further reduce the size of the particles as seen by electron microscopy. Detailed studies of size distributions of particles after emulsification for different times with different chamber dimensions and at different lipid concentrations are underway using quasi-elastic light scattering [8]. In the studies made so far, however, it is clear that by varying

TABLE I

CAPTURE OF CYTOSINE ARABINOSIDE BY MICROEMULSIFIED LIPOSOMES (MEL), MULTILAMELLAR VESICLES (MLV), SMALL UNILAMELLAR VESICLES (SUV) AND REVERSE PHASE EVAPORATION (REV) LIPOSOMES

All lipid compositions were PG/PC/Chol (0.1 : 0.4 : 0.5, mole ratio).

Type of liposome	Initial lipid concentration (μmol/ml)	Recycling time (min)	% Capture [a]	Liter aqueous/ mole lipid [b]
MEL	60	2	6.3	1.03
MEL	60	10	5.0	0.83
MEL	180	2	17.4	0.97
MEL	180	10	16.0	0.89
MEL	300	10	78.0	0.73
MEL	300	30	73.9	0.69
MEL	300	60	74.6	0.69
		Extrusion filter size		
SUV	60	–	3.2	0.54
SUV	180	–	8.4	0.47
MLV	60	Unextruded	10.7	1.79
MLV	60	0.2 μm	9.0	1.50
MLV	180	Unextruded	26.9	1.49
MLV	180	0.2 μm	24.9	1.38
REV	60	Unextruded	45.7	7.6
REV	60	0.1 μm	9.6	1.6
REV	180	Unextruded	50.0	2.8
REV	180	0.1 μm	19.2	1.1

[a] Amount of Ara-C entrapped in liposomes in preparation as a percent of total Ara-C at start.

[b] Calculated on the basis that Ara-C is entirely trapped in the aqueous space and is not bound by lipid.

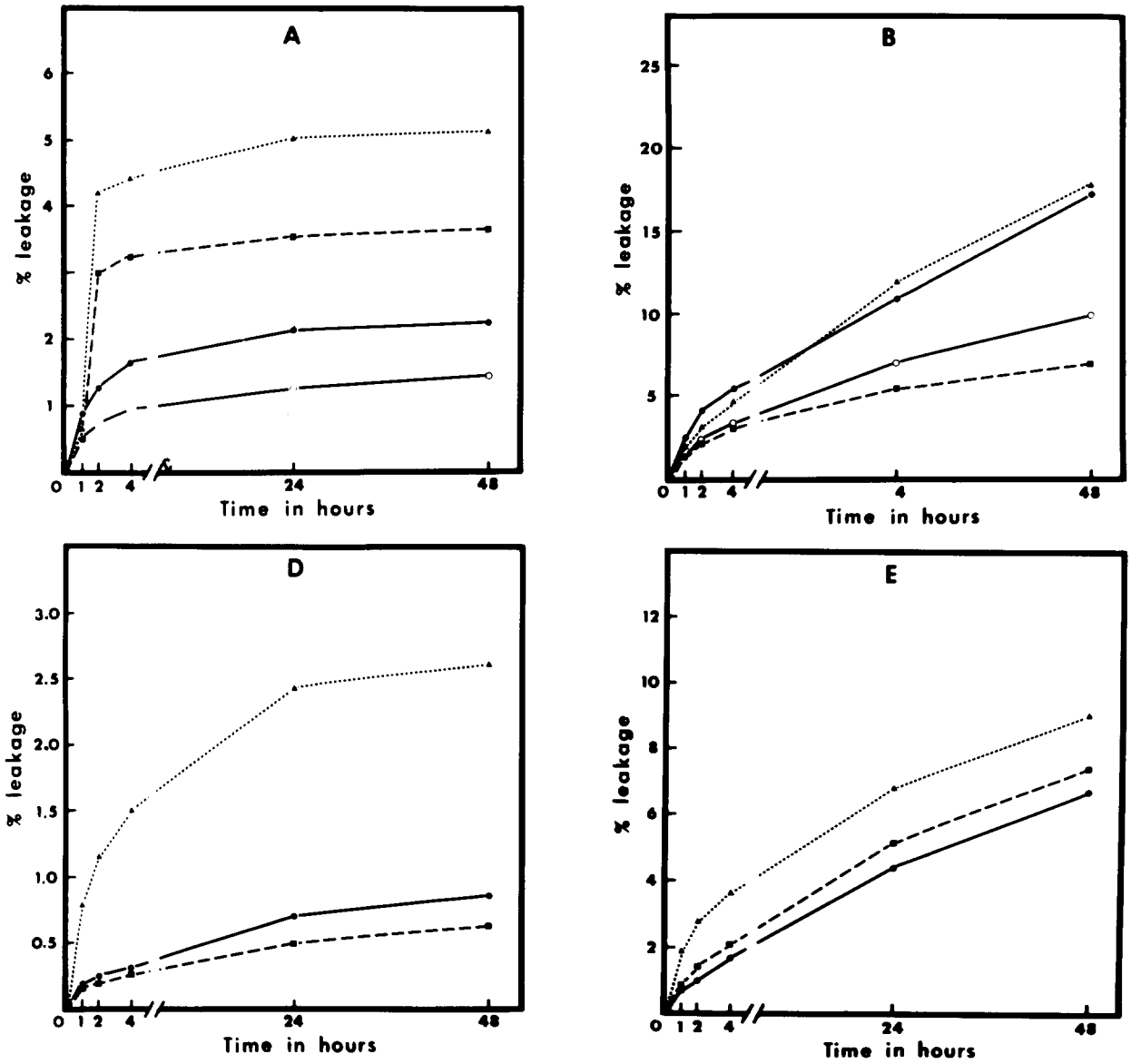
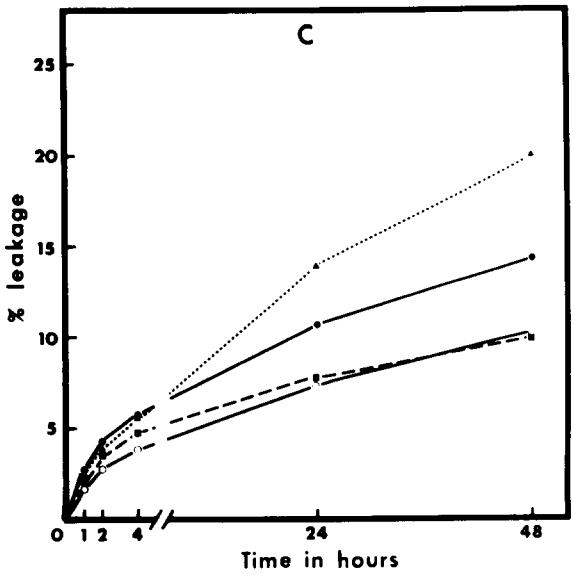
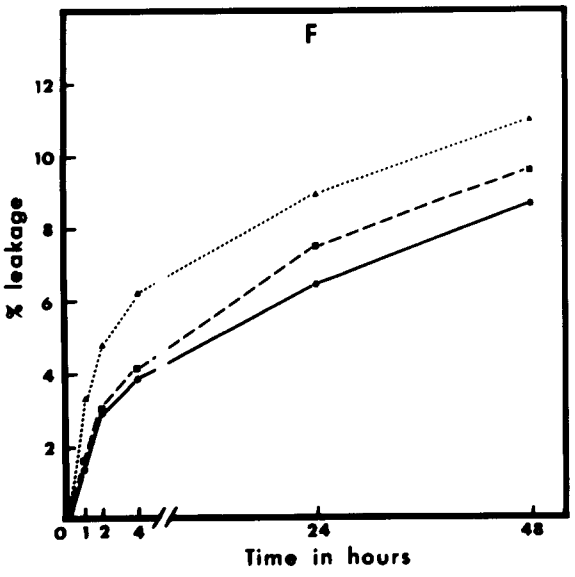


Fig. 3. Permeability of liposomes with time. (A–C) PG/PC/Chol (0.1 : 0.4 : 0.5), 60 μmol/ml. (D–F) PG/PC/Chol (0.1 : 0.4 : 0.5), 300 μmol/ml. (A) 4°C; (B) 37°C; (C) 37°C plus serum. ▲···▲, small unilamellar vesicles; ■– – –■, multilamellar vesicles; ●———●, microemulsified liposomes 10 min; ○———○, microemulsified liposomes 2 min. (D) 4°C; (E) 37°C; (F) 37°C plus serum. ▲···▲, microemulsified liposomes 60 min; ●———●, microemulsified liposomes 30 min; ■– – –■, microemulsified liposomes 10 min.

the time of microemulsification, the size distribution of the particle can be regulated. Under similar processing conditions, the size distribution of particles produced is reproducible.

Table I compares the aqueous capture efficiency of microemulsified liposomes with small unilamellar vesicles, multilamellar vesicles and reverse phase evaporation liposomes. It can be seen that the % capture of Ara-C increases when the initial lipid concentration is raised from 60–180 μmol/ml for all types of liposomes. Multilamellar vesicles, small unilamellar vesicles and reverse phase evaporation liposome preparations are impossible to handle at initial concentrations of 300 μmol/ml, whereas the Microfluidizer™ can prepare liposomes efficiently at this concentration. As





can be seen from Table I, the capture of Ara-C by microemulsified liposomes at 300 μmol/ml lipid is higher than that of 0.1 μm reverse phase evaporation liposomes prepared at 180 μmol/ml. The capture of Ara-C by unextruded, hence very heterogeneous, reverse phase evaporation liposomes at 180 μmol/ml is approx. 50% which is still less than the maximum capture for microemulsified liposomes. The smallest filter through which multilamellar vesicles can be extruded easily is 0.2 μm thus limiting multilamellar vesicle preparations to approx. 0.2 μm diameter minimum size. Small unilamellar vesicles liposomes capture less aqueous phase than multilamellar vesicles at equal initial lipid concentrations and are in the size range of 0.02–0.03 μm diameter. The aqueous phase/lipid ratios given in Table I indicate that the aqueous space capture efficiency for microemulsified liposomes is intermediate between small unilamellar vesicles and multilamellar vesicles or reverse phase evaporation liposomes. This last finding suggests microemulsified liposomes have a size greater than that of small unilamellar vesicles but smaller than that of easily prepared multilamellar vesicles or reverse phase evaporation liposomes. Studies are underway to determine the number of lipid lamellae in microemulsified liposomes.

Figs. 3A, B, C compare the permeability of microemulsified liposomes to Ara-C with standard multilamellar vesicles and small unilamellar vesicles prepared at equal lipid/aqueous volume ratios. The data show that microemulsified liposomes were less permeable than small unilamellar vesicles or multilamellar vesicles at 4°C. Most of the differences in permeability over a 48 h period could be accounted for by a rapid initial permeability phase, noted previously for multilamellar vesicles and small unilamellar vesicles [5], that was not seen with microemulsified liposomes. Microemulsified liposomes (10 min) were more permeable than microemulsified liposomes (2 min). At 37°C in the absence of serum, microemulsified liposomes (10 min) showed similar permeability to small unilamellar vesicles; and microemulsified liposomes (2 min) showed similar permeability to multilamellar vesicles. In the presence of serum, small unilamellar vesicles were slightly more permeable than microemulsified liposomes (10 min) whereas multilamellar vesicles and microemulsified liposomes (2 min) had similar permeabilities.

Permeability measurements were also made of microemulsified liposome preparations in which the starting lipid concentration was 300 μmol/ml (approx. 20% w/v) (Figs. 3D, E, F). There was no significant difference between Microfluidizer™ preparations processed for 10 or for 30 min. It can be seen that at 37°C, and 37°C plus serum, the permeabilities of both preparations were comparable. At 4°C the microemulsified liposomes 300 μmol/ml preparations were less permeable than the 60 μmol/ml preparations.

Discussion

The results suggest that microemulsified liposomes have properties similar to small extruded multilamellar liposomes. However, compared with normal multilamellar vesicle preparations, use of the Microfluidizer™ has the advantage that the size distribution is uniform, without the need for terminal filtration, and the liposomes are smaller than the smallest easily prepared multilamellar vesicles. Extrusion [6,7] procedures require use of various sized filters and have associated problems of clogging, sterility and flow. In addition, extrusion of multilamellar vesicles preparations through 0.1 μm filters or smaller is more or less impossible. In addition, the Microfluidizer™ system can work at considerably higher concentrations of lipid than are possible with extruded multilamellar vesicle preparations. This results in reduced loss of entrapped material and a higher final concentration of drug in microemulsified liposomes, without the need for concentration procedures required in preparing multilamellar vesicles.

Compared with the reverse phase evaporation liposome procedure, the capture by microemulsified liposomes of aqueous space marker per μmol of lipid is similar or less at equal initial lipid concentrations. However, the Microfluidizer™ procedure can, at high lipid concentrations, capture as much or more of the drug on a percentage basis. Unlike the reverse phase evaporation procedure liposomes the Microfluidizer™ process does not involve the use of relatively expensive organic solvents in the presence of the aqueous phase. In addition, microemulsified liposomes can be prepared by a continuous process rather than the batch process required for reverse phase evaporation liposomes. The microemulsifier device can handle any quantity of lipid/drug suspension depending only on need.

The permeability data shows that microemulsified liposomes are at least as stable as multilamellar vesicles under similar environmental conditions and that permeability increases as size is reduced, possibly related to decreases in the number of lamellae.

Preliminary studies have indicated that microemulsified liposomes (10 min) aggregate much less than small unilamellar vesicles, multilamellar vesicles or reverse phase evaporation liposomes of similar compositions over a six month period or more. Perhaps this is due to the attainment of stable equilibrium sizes of the microemulsified liposomes during microemulsification and recycling.

Microemulsified liposomes of sizes intermediate between that of small unilamellar vesicles and extruded multilamellar vesicles could be useful for various in vitro and in vivo purposes. As has been pointed out, in vivo the smaller the liposomes the greater the chances that liposomes can escape reticuloendothelial uptake and reach tissues outside of the vasculature [4]. In particular, the cut-off size range of endothelial layer fenestrations in tissues such as the liver may lie between 0.1 and 0.2 μm [9] so that liposomes less than 0.1 μm in size but with high efficiency of capture may be particularly useful for delivery of drugs and other agents.

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