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A procedure for the efficient entrapment of drugs in dehydration-rehydration liposomes (DRVs)

Gregory Gregoriadis, Helena da Silva and Alexander T. Florence

Centre for Drug Delivery Research, The School of Pharmacy, University of London, 29/39 Brunswick Square, London WC1N 1AX (U.K.)

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Summary

Radiolabelled maltose and tetanus toxoid were each entrapped in multilamellar dehydration-rehydration vesicles (DRVs) composed of egg phosphatidylcholine (up to 66 μmol) and equimolar cholesterol. Solute-containing DRVs were microfluidized to smaller vesicles (to a mean diameter of around 100 nm, as measured by photon correlation spectroscopy) which retained 10–100% of the originally entrapped solute. Solute retention was found to be dependent on the number of microfluidization cycles, the medium in which microfluidization was carried out and on whether or not unentrapped solute was removed before processing. Under appropriate conditions, vesicles with mean diameters of less than 200 nm were produced, retaining about 35–78% of the originally entrapped solute. These would be suitable for in vivo use where small vesicle size and an augmented entrapped solute to liposomal lipid mass ratio are required.

Introduction

High-yield entrapment of solutes in liposomes using minimal amounts of lipids is a prerequisite for their effective use as a drug carrier (Gregoriadis, 1988) as a high entrapped drug to lipid mass ratio will reduce both the cost of formulations and the risk of lipid-induced toxicity following injection. Although at least two methods meeting such requirements have been developed (Szoka and Papahadjopoulos, 1978; Kirby and Gregoriadis, 1984), they both produce large liposomes with diameters approaching the micrometer size range. On intravenous injection these larger liposomes exhibit short half-lives in the blood circulation (Senior et al., 1985) and are, therefore, unsuitable for maintaining increased concentrations of solutes (eg. haemoglobin) within the vascular system or for ligand-mediated or other modes of targeting to accessible cells (Gregoriadis, 1988). Furthermore, intramuscularly injected large liposomes are unable to reach the regional lymph nodes efficiently (Tumer et al., 1983) and to deliver vaccines and other agents to these sites (Gregoriadis, 1988). Such tasks, however, can be carried out (Tumer et al., 1983; Senior et al., 1985; Spanzer et al., 1986; Allen and Chonn, 1987; Gabizon and Papahadjopoulos, 1988; Chow et al., 1989) by smaller vesicles of about 200 nm or less in diameter.

Correspondence: G. Gregoriadis, The School of Pharmacy, University of London, 29/39 Brunswick Square, London WC1N 1AX, U.K.

Unfortunately, these tend to exhibit low entrapment yield when prepared by conventional techniques (Mayhew et al., 1984). Whilst substantial entrapment values can be obtained (Mayhew et al., 1984) for small liposomes prepared by extrusion through polycarbonate filters or by microfluidization, large amounts of lipid (180 and 300 μmol/ml, respectively) have been employed to achieve this.

It was previously shown (Kirby and Gregoriadis, 1984; Gregoriadis et al., 1987) that entrapment yields up to 80% of the starting material can be obtained in large multilamellar vesicles using modest amounts of lipid (up to 66 μmol total) by the dehydration-rehydration procedure producing what we call DRVs. We now report that microfluidization of such vesicles produces liposomes down to 100 nm in diameter which retain 10–100% of the originally entrapped solute. Solute retention depends on the extent of microfluidization, the medium in which microfluidization is carried out, and on whether or not, before processing, DRVs are previously separated from the unentrapped solute.

Materials and Methods

The source and grades of egg phosphatidylcholine (PC) and cholesterol have been reported elsewhere (Kirby and Gregoriadis, 1984). Maltose was from Sigma (London) and immunopurified tetanus toxoid from Wellcome Biotechnology (Beckenham). D-[U-^{14}C]Maltose (20 GBq, per μmol) was purchased from Amersham International (Amersham, U.K.) and toxoid labelled (specific activity 5.43 mBq) with ^{125}I as described (Gregoriadis et al., 1987).

Preparation of liposomes

Solute-containing DRV liposomes were prepared by the procedure discussed before (Kirby and Gregoriadis, 1984; Gregoriadis et al., 1987). Briefly, small unilamellar vesicles (SUV) made in distilled water from equimolar PC (16.5, 33, 66 or 132 μmol) and cholesterol were mixed with an equal volume (2.0 ml) of maltose (7.2 mg) or tetanus toxoid (7.2 mg) dissolved in PBS (0.44 mM sodium phosphate, 2.7 mM potassium chloride and 0.14 M sodium chloride, pH 7.4) and supplemented with traces (4.9×10^5–8.7×10^5 dpm) of the respective radiolabelled solutes. Following dehydration and controlled (Kirby and Gregoriadis, 1984) rehydration to form solute-containing DRV, the suspensions (2.0 ml) were each divided into two equal portions. One portion was centrifuged at $20\,000 \times g$ for 30 min, the pellet washed three times with PBS and suspended in 20 ml of the same buffer (washed DRV) for microfluidization. Entrapment of solutes was estimated by the assay (Kirby and Gregoriadis, 1984; Gregoriadis et al., 1987) of ^{14}C or ^{125}I radioactivity in the suspension, and results expressed as percentage of the solute originally present. The other portion (containing a mixture of entrapped and unentrapped solute) was also diluted in 20 ml PBS (unwashed DRV) and then microfluidized. In some experiments, PBS was replaced by distilled water in all steps involved in the entrapment of solutes in DRV and microfluidization of washed or unwashed preparations.

Microfluidization of liposomes

Washed and unwashed DRV (20 ml each) were passed for 1.8, 3.5, 5.2, 7.1 and 10.6 full cycles through a Microfluidizer 110^{TM} kindly provided by Microfluidics Corp. (Newton, MA, U.S.A.). The pressure gauge was set at 60 psi throughout the procedure to give a flow rate of 35 ml per min. At the end of each of the 20 ml cycle intervals, samples containing maltose were dialysed exhaustively against distilled water or PBS, and the toxoid containing vesicles centrifuged for 30 min at $20\,000 \times g$ (1.8 and 3.5 cycles) or $35\,000 \times g$ (5.2, 7.1 and 10.6 cycles), the pellets washed twice in PBS or water and resuspended in 1.0 ml of the respective solvents. In the case of the toxoid, the supernatants obtained on centrifugation were passed (Senior et al., 1985) through Sepharose 4B columns; very little (less than 3%) of the solute in the centrifuged samples eluted in the liposome form. The extent to which washed and unwashed DRVs retained their solute content after microfluidization was estimated as follows: for washed DRVs, radioactivity retained after dialysis or recovered in pellets after centrifugation was ex-

pressed as a percentage of the total amount in the sample before dialysis or centrifugation. Solute retention values thus obtained correspond to the percentage of the original entrapment values in the preparations before microfluidization. For unwashed DRV, the percentage of radioactivity retained after dialysis or centrifugation was expressed as percentage of the original entrapment values.

Measurement of vesicle size

Particle size distributions were measured by photon correlation spectroscopy of samples diluted in either water or PBS, using a Malvern Model 4700 apparatus (Malvern Instruments Ltd., Malvern, U.K.) equipped with a 25 mW helium/neon laser. Mean diameters and size distributions are obtained: the *z*-average mean diameter, polydispersity factor and cumulative percentage mass and number undersize data were recorded as a function of the number of microfluidization cycles. The performance of the instrument was checked with monodisperse polystyrene latex suspensions (Polysciences, U.K.) and mixtures of such latex samples to verify the ability of the system to accurately measure polydisperse or bidisperse systems.

Results and Discussion

The effect of DRV microfluidization on solute retention

As expected from previous work (Kirby and Gregoriadis, 1984; Gregoriadis et al., 1987), entrapment of solutes in dehydration-rehydration liposomes was efficient, ranging from 19.2 to 66.1% of the starting material and dependent on the amount of phospholipid used (see legends to Figs 1–3). There was no significant difference in entrapment values in preparations made in distilled water or PBS (not shown). Retention values (quoted as a percentage of original entrapment values) by solute-containing DRVs microfluidized for up to 5.2 cycles in the presence of PBS are shown in Figs 1 and 2. It is apparent that retention values by both washed and unwashed DRV decrease or remain the same as the number of

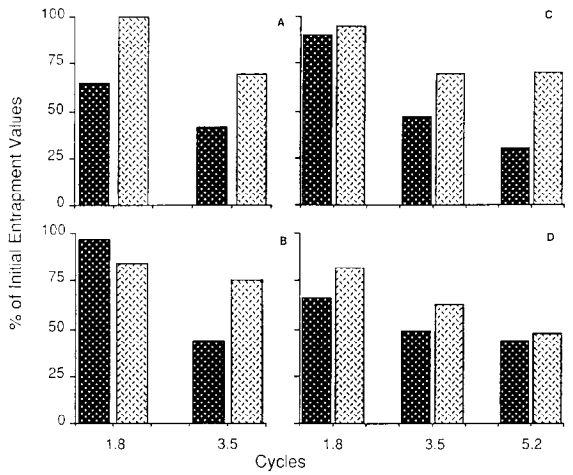

Fig. 1. Solute retention values by DRVs following microfluidization. ^{14}C-labelled maltose-containing DRVs composed of equimolar PC and cholesterol were microfluidized in the washed (filled bars) or unwashed (shaded bars) forms in the presence of PBS for up to 5.2 cycles. Amount of liposomal phospholipid passed through the microfluidizer was 8.25 (A), 16.5 (B), 33 (C) and 66 (D) μmol. Values denote % retention of originally entrapped radioactive solute. Original entrapment values were 27.1 (A), 33.6 (B), 66.1 (C) and 65.7% (D) of the material used for entrapment.

cycles increases (see also Fig. 3). Thus, values of 80–100% (maltose) after 1.8 cycles become 40–65% after 5.2 cycles (Fig. 1), whereas values for the toxoid (unwashed DRV) remain virtually unchanged (at around 75%) (Fig. 2). It is conceivable that during microfluidization, a process entailing breakage of the integrity of the vesicle membrane and consequent leakage of entrapped solute occurs, large molecules such as proteins leak at slower rates than smaller molecules. Furthermore, no apparent difference in solute retention was observed for preparations containing varying amounts of PC (8.25–66 μmol) (Figs 1 and 2). Figs 1 and 2 also indicate that, generally, a greater proportion of solute (maltose or toxoid) is retained by unwashed vesicles (i.e. preparations microfluidized in the presence of unentrapped solute) than by washed DRV. The presence of unentrapped solute during microfluidization diminishes solute leakage perhaps by reducing the osmotic rupture of the vesicles and by reducing initial concentration gradients across the membrane.

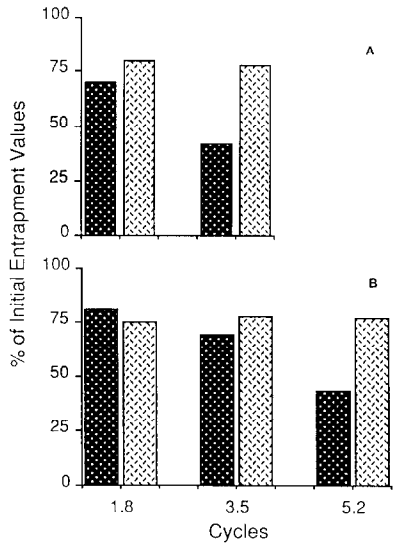

Fig. 2. Solute retention values by DRV following microfluidization. ^{125}I-labelled tetanus toxoid-containing DRVs composed of equimolar PC and cholesterol were microfluidized in the washed (filled bars) or unwashed (shaded bars) forms in the presence of PBS for up to 5.2 cycles. Amount of liposomal phospholipid passed through the microfluidizer was 8.25 (A) and 33 (B) μmol. For other details see legend to Fig. 1. Original entrapment values were 19.2 (A) and 56% (B) of the material used for entrapment.

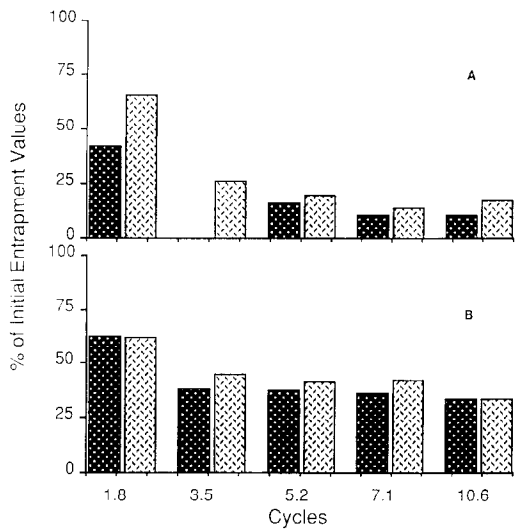

Fig. 3. The effect of medium during microfluidization on solute retention by DRV. ^{14}C-labelled maltose-containing DRVs composed of equimolar PC and cholesterol were microfluidized in the washed (filled bars) and unwashed (shaded bars) form in the presence of distilled water (A) or PBS (B) for up to 10.6 cycles. Amount of liposomal phospholipid passed through the microfluidizer was 33 μmol. For other details see legend to Fig. 1. Original entrapment values were 55.2 (PBS) and 54.3% (water) of the material used for entrapment.

The mean diameter of microfluidized DRVs measured by dynamic light scattering after a given cycle interval depended on whether the DRVs were suspended in distilled water or PBS. We therefore attempted to determine the effect of the two media on solute retention during microfluidization. Results in Fig. 3 indicate that much more maltose is retained by DRVs in the presence of PBS and similar results (not shown) were obtained with toxoid containing DRVs under identical conditions. Such an effect of the presence of PBS in the milieu could be attributed to its reduction of osmotic shock expected (Kirby and Gregoriadis, 1984) to occur on dilution of solute-containing DRV in hypotonic media.

The effect of microfluidization on vesicle size

The mean diameters of washed and unwashed maltose-containing DRV microfluidized in water or PBS (from the experiment described in Fig. 3), measured by photon correlation spectroscopy, are presented in Table 1. They show that, in agreement with findings by others (Talsma et al., 1989), a smaller vesicle size was achieved for preparations processed in water than in PBS. Such dif-

TABLE 1

z average mean size (nm) of microfluidized DRV [a]

DRV	Cycles				
	1.8	3.5	5.2	7.1	10.6
Washed					
Water	463.5	149.9	115.0	121.9	114.7
PBS	447.4	198.6	168.1	159.5	155.7
Unwashed					
Water	473.9	132.9	116.9	116.6	101.9
PBS	456.3	186.2	186.7	169.8	159.9

[a] Maltose-containing washed or unwashed DRV (33 μmol PC) were microfluidized in the presence of water or PBS for up to 10.6 cycles and samples measured for vesicle size (diameter in nm) by dynamic light scattering (photon correlation spectroscopy). Polydispersity indexes ranging from 0.503 to 0.653 (water) and 0.517 to 0.653 (PBS) were similar to those obtained with some of the lipid compositions of liposomes employed by Talsma et al. (1989).

ferences in sizes, appearing after the 1.8 cycle interval, could be attributed (Deamer and Uster, 1983) to vesicle aggregation induced by the presence of salts in the PBS medium, but also might be due to the increased lipophilicity of the lipids in the presence of electrolyte. Reduction in the size of all preparations tested was considerable after the initial 1.8 cycle interval. Subsequently, sizes levelled off to reach minimal values of 100–160 nm by 10.6 cycles depending on the medium (water or PBS) (it is of interest that a pattern similar to that of sizes in Table 1 was observed for solute retention with increasing cycle intervals (Fig. 3), with values also levelling off after 3.5 cycles). Mean number diameter and mass diameter distribution data (Figs 4 and 5) from the experiments recorded in Table 1 revealed a bimodal distribution of sizes in which there is an increasing number of small vesicles as the number of microfluidization cycles increases beyond the first few cycles; the mean number diameter of the sample, prepared in water (50 mg maltose/water) is of the order of 300 nm after 1.8 cycles, with a few vesicles with diameters up to 1 μm in diameter. The larger diameter vesicles disappear rapidly on increasing cycling, so that after 10.6 cycles in water, a single narrow diameter (around 100 nm) distribution is obtained (Fig. 4) whereas in PBS the bimodal pattern is retained (Fig. 5). However, the vesicle size distribution does not exhibit the

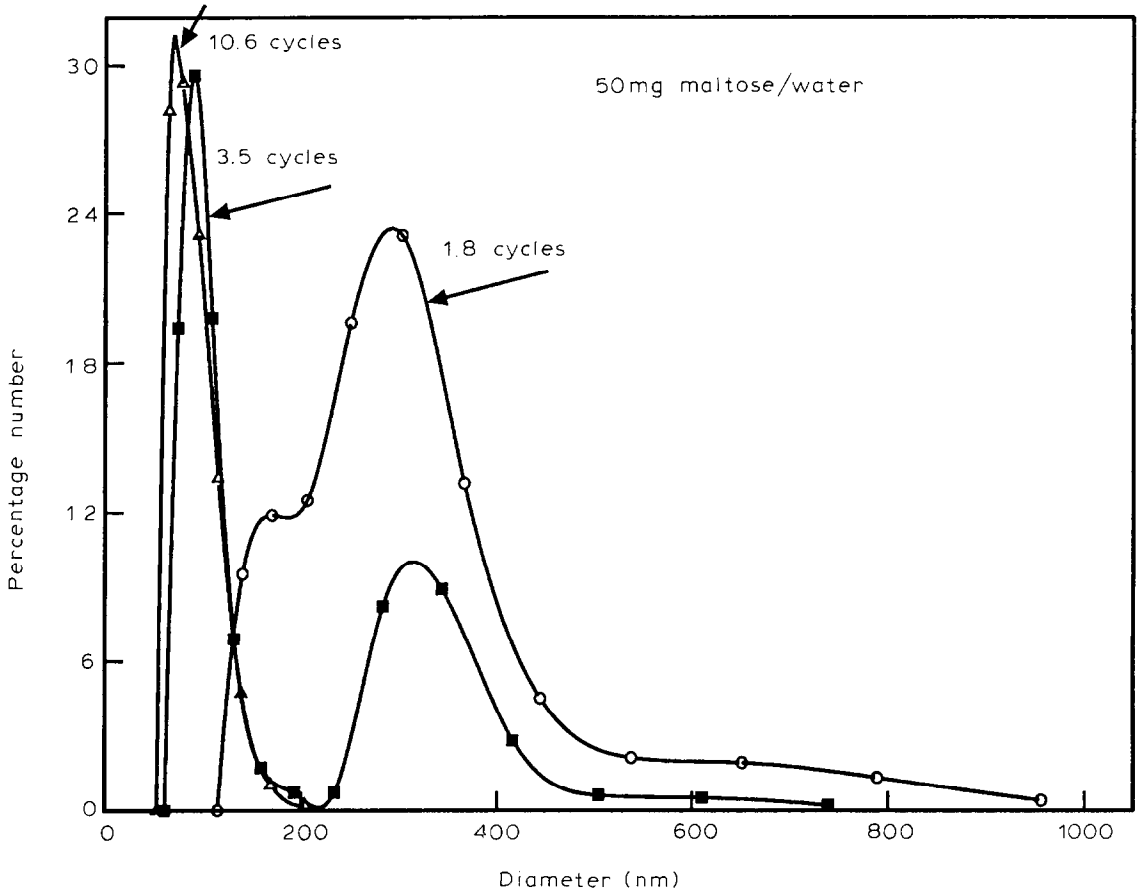

Fig. 4. The size distribution of vesicles containing maltose prepared in the presence of water as a function of the number of cycles through the Microfluidizer, showing the progression to a distribution with a mean size of approx. 100 nm after 10.6 cycles.

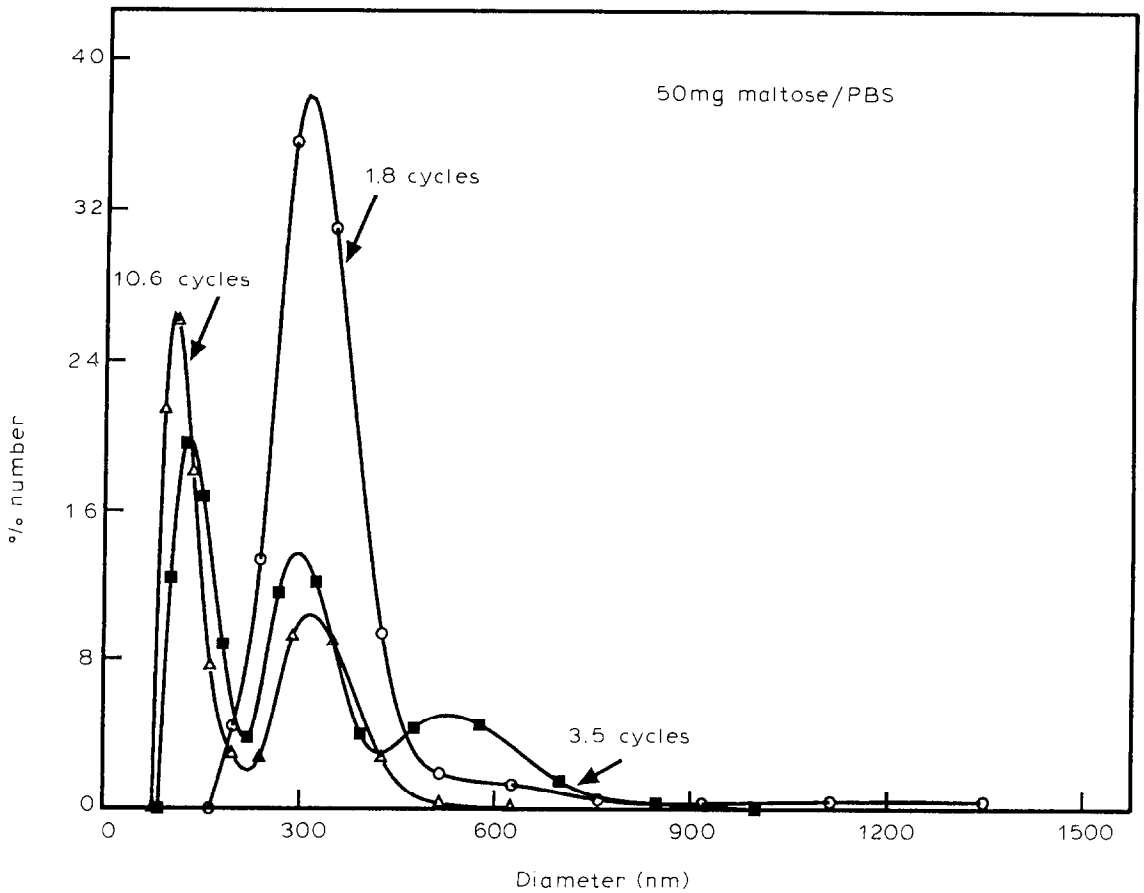

Fig. 5. The size distribution of vesicles containing maltose prepared in the presence of phosphate-buffered saline (PBS) as a function of the number of Microfluidizer cycles. After 10.6 cycles, the number of vesicles with diameters greater than 300 nm is decreased but a biphasic distribution is still maintained.

gradual downward drift that occurs on, say, sonication but a transfer of sizes from one size band to another. In PBS, some vesicles undoubtedly flocculate so that there remains a percentage of vesicles with a high apparent diameter. Fig. 6 illustrates the distribution of diameters by mass. Here is clear evidence of the marked narrowing of the polydispersity of the vesicles. Coupled with the data from Fig. 4, this figure clearly shows the narrow distribution of sizes which is achieved after 10 cycles. The results reported by Lidgate and co-workers (1989) show that in preparing parenteral emulsions of squalene by microfluidization, the particle size after 9–10 cycles is in the range of 0.07–0.2 μm which represents a much narrower distribution than is achieved with other technologies.

In conclusion, microfluidization of solute-containing DRVs in PBS and the presence of unentrapped solute produce vesicles with sizes of less than 200 nm suitable for tasks discussed earlier. Such vesicles retain, under the present conditions, 35–78% of the originally entrapped solute (Figs 1–3). Compared to procedures (Mayhew et al., 1984) which employ much larger amounts of lipid to achieve efficient entrapment, the present approach provides preparations with augmented solute to lipid mass ratios and should also be more economical. The narrow distribution of final vesicle sizes must reflect the nature of the produc-

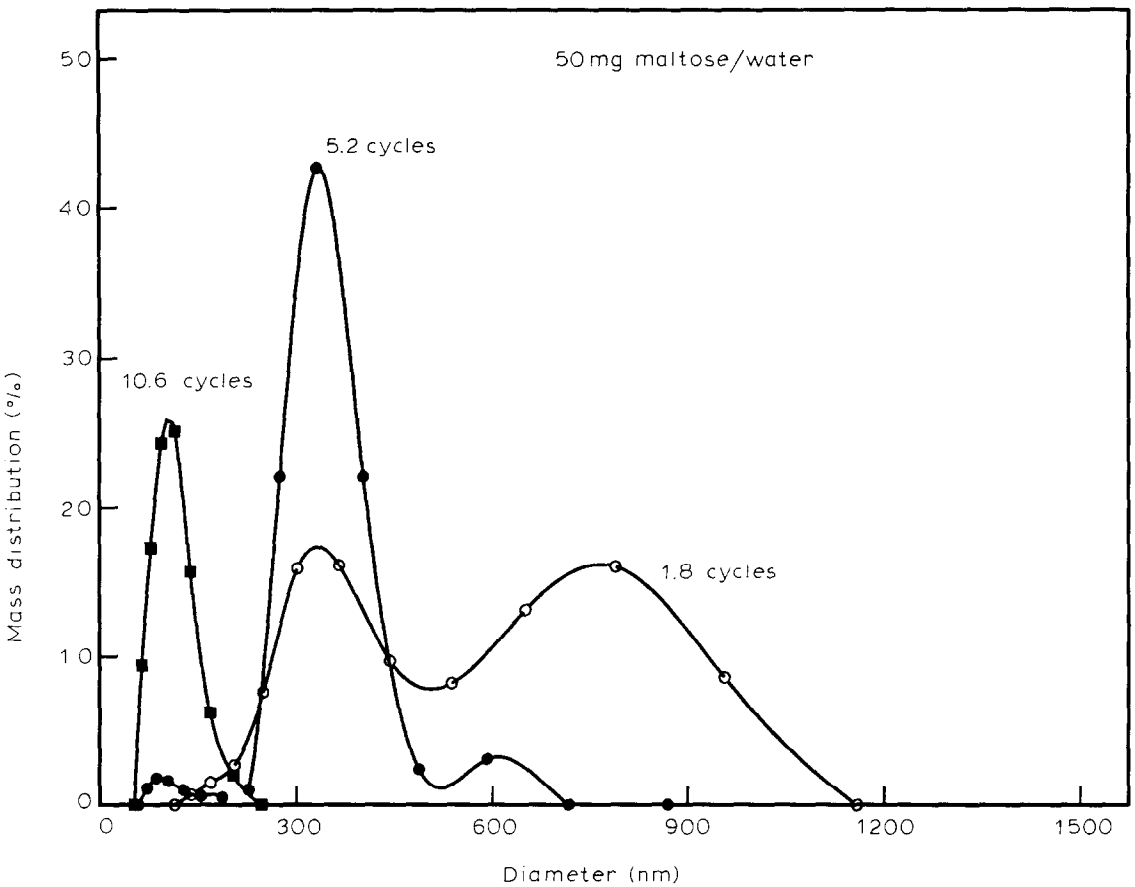

Fig. 6. The mass distribution of maltose-containing vesicles which are the subject for Fig. 4 as a function of fluidization cycles. These data along with the number distribution data of Fig. 4 confirm the narrow distribution sizes after 10.6 cycles.

tion process. Whereas sonication disrupts vesicles in a random mode producing a wide range of diameters, microfluidization distorts large and flexible vesicles by extrusion through a capillary, thereby increasing the number of vesicles of similar size which 'bud off' from simple parent vesicles.

Acknowledgements

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