LARGE-SCALE BLOOD SUBSTITUTE PRODUCTION USING A MICROFLUIDIZER

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SUMMARY

Microfluidization has been tested as a way to disperse phospholipids in aqueous hemoglobin solutions. Spherical and stable liposomes of 2 to 3 µm were obtained. Lipid incorporation (up to 85 %) and hemoglobin encapsulation (up to 15 %) in liposomes have been improved with respect to previous investigations. However, results show that a more efficient dispersion system using lower concentrations of lipid is required to obtain a high liposome hemoglobin concentration (limited actually to 150 g/l) and an economically and biologically suitable process for artificial blood production at large scale.

INTRODUCTION

Blood is an essential for mammalian life and blood transfusions have become an everyday life-saving measure. However, problems associated with blood transfusions such as transmission of diseases, hemolytic transfusion reactions, circulatory overload, embolism and coagulation disturbances, have made the development of an acceptable blood substitute a desirable goal [Djordjevich, 1980]. It appeared interesting to develop a synthetic blood replacement that matched the properties of whole human blood to avoid the problems associated with blood transfusions. The encapsulation of concentrated hemoglobin solutions within lipid vesicles is one of the most promising approaches [Djordjevich, 1980]. The similarity of liposomes to natural biostructures should provide a compatibility and safety profile of great interest to the medical community [Mayhew, 1985].

Classical methods used for producing liposome-encapsulated hemoglobin, such as the lipid film method and sonication [Rabinovici, 1990; Kirby, 1984a], faced important problems: heterogeneity in both size and form of liposomes, instability of the liposomal membrane conducting to leakage of the encapsulated hemoglobin, very small production scale and a low encapsulation yield for both encapsulated material and lipid included in the liposomal membrane [Vivier, 1991]. The production of such liposome-encapsulated hemoglobin is then very expensive and only feasible on a laboratory scale

A relatively new alternative for the production of liposomes consists in the dispersion of lipid sin an aqueous solution with the help of a microfluidizer [Cheng, 1987]. Microfluidization is a technique which utilizes the force of two streams of lipid suspension colliding with each other under high pressure to reduce the vesicle size. Vemuri [1990] observed that the use of a microfluidizer leads to an homogeneous liposome size distribution and a higher stability. The size of liposomes may be controlled by adjusting the inlet air pressure and the number of recycles [Cheng, 1987].

The present paper proposes an evaluation of the potential of microfluidization for large scale artificial red cell production. The engineering approach has been favoured, looking more specifically to yields or scale of production.

MATERIALS AND METHODS

1. Hemoglobin solutions

Hemoglobin solutions were prepared from human blood by washing erythrocytes three times with 0.9 % NaCl solutions and centrifuging for 10 min at 4,000 g (4 °C). The erythrocytes were broken down using an homogenizer (Ultra Turrax, Janke Kunkel, Staufen, Germany). Eight cycles of agitation (30 s) and rest (30 s) were applied to the erythrocyte pellet in an ice bath to avoid heating of the hemoglobin solution. Cell membranes were removed by centrifugation at 16,000 g for 30 min. Hemoglobin concentrations, measured by spectrophotometry (Spectronic 601, Bausch and Lomb, USA) at a wavelength of 230 nm and a molar extinction coefficient of 142,000 [Fasman, 1976], were ranging from 400 to 460 g/l.

2. Phospholipids

The lipid used for liposome formation contained a minimun of 99 % of hydrogenated soy phosphatidylcholine (Phospholipon 100 H, Nattermann Phospholipid, Köln, Germany). The hydrogenated form has been selected to avoid oxidation. In some case, cholesterol (Sigma Chemicals, St-Louis, MO) was added to the phospholipids prior to liposome formulation.

3. Microfluidization and microfluidizer

Figure 1 shows a schematic diagram of the microfluidizer. The suspension to be microfluidized is introduced in the inlet reservoir (1). The suspension is pumped at high pressure (2) through a 5 µm filter (3). The flow is divided in two streams which react at ultrahigh velocities (> 500 m/s) in precisely defined microchannels within the interaction chamber (4) [Mayhew, 1985]. The microfluidized suspension is collected at the exit (5) and may be recycled. The microfluidizer used in this work (M110, Microfluidics, Newton, MA, USA) was composed of two interaction chambers (4).

4. Preparation of liposomes

Phosphatidylcholine (24 g) and hemoglobin (100 ml, approx. 400 g/l) were homogenized by using an Ultra Turrax (Junke Kunkel, Staufen, Germany). The resulting mixture was heated to 50 °c under agitation and processed through the microfluidizer, preheated by passing hot water through the system. Liposome suspension were collected at the exit, recirculated batchwise or treated to isolate the liposomes. The unencapsulated hemoglobin solution was removed by washing the liposomes three times with isotonic PBS 0.9 % at pH 7.4 (138 mM NaCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, 3 mM KCl) and centrifuging for 30 min at 16 000 g.

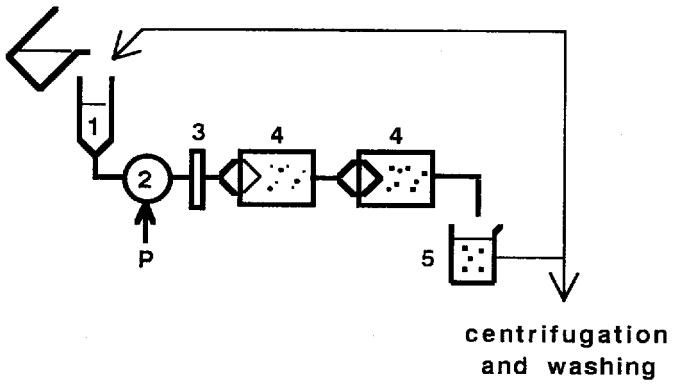

FIGURE 1: Diagram of microfluidizer

5. Size distribution of liposomes

Liposomes size distribution was determined by dynamic light scattering analysis (Nicomp 370 Instruments, Particle Sizing Systems, Santa Barbara, CA, USA). This technique measures the fluctuations in scattered light intensity generated by the diffusion of vesicles in the solution [Nicoli, 1990]. The resulting size distributions are expressed in relative intensity and are relatively similar to the volume distribution. Prior to analysis, the sample containing the liposomes were diluted in phosphate buffer (PBS 0.9 %, pH 7.4) and filtered through a 30 μm nylon filter.

6. Microscopy

Liposomes were first visualized with an optical microscope (Carl Zeiss, Jena, Germany) at a magnification of 1500 X. The individual characteristics of the liposomes (lamellae, leak of liposome content etc.) were studied by electron microscopy Droplets of liposome suspensions were deposited on carbon-coated copper grids, stained with 2 % potassium phosphotungstate (pH 7.0), and examined in a Philips EM 300 electron microscope operated at 60 kV. Magnification was monitored with catalase crystals [Luftig, 1967].

7. Quantification of hemoglobin encapsulated in liposomes (η_h)

The hemoglobin content of microfluidized liposomes was obtained from the determination of the nitrogen content of proteins (Kjeldahl's method) [Plummer, 1971]. The pellet of washed liposomes was dried overnight under vacuum at 90 °c. The weight of the sample was recorded, Q_v. A known weigth of the dried material was digested with concentrated sulphuric acid in the presence of selenium dioxide by heating for 18 hr. Ammonium sulphate was then formed from the protein nitrogen. The mixture was steam distilled and the ammonia released was passed into boric acid which was then back titrated with standard acid.

The fraction of hemoglobin encapsulated in liposomes or encapsulation yield, η_h, is given by:

$$\eta_h = Q_{hv} / Q_{ho} \quad (1)$$

where Q_{hv} and Q_{ho} are the amount of hemoglobin in the vesicle (liposome) and in the initial suspension, respectively.

8. Quantification of the lipid incorporated in liposomes (η_l)

The amount of lipid included within the liposomal membrane, Q_{lv}, is estimated by the difference between the dried liposome weight, Q_v, and the amount of encapsulated hemoglobin, Q_{hv}:

$$Q_{lv} = Q_v - Q_{hv} \quad (2)$$

The fraction of lipids included within the liposomal membrane or lipid yield, η_l, is calculated as follows:

$$\eta_l = Q_{lv} / Q_{lo} \quad (3)$$

where Q_{lo} is the amount of lipids introduced in the system.

9. Liposome hemoglobin concentration ($[Hb]_v$)

The volume of hemoglobin solution in liposomes, V_{hv}, is given by:

$$V_{hv} = \eta_h \ V_{ho} \quad (4)$$

where V_{ho} is the total volume of hemoglobin solution. The volume of lipids in vesicles, V_{lv}, is equal to:

$$V_{lv} = \eta_l \ Q_{lo} / \rho_l \quad (5)$$

where ρ_l is the specific mass of lipids in aqueous suspension. The aqueous fraction of liposomes, α, is then calculated by:

$$\alpha = V_{hv} / V_{lv} \quad (6)$$

Assuming a concentration of hemoglobin, $[Hb]_o$, equal in the core of liposomes and in the aqueous solution, the mean hemoglobin concentration in the liposome volume, $[Hb]_v$, is given by:

$$[Hb]_v = [Hb]_o \ \alpha \quad (7)$$

10. Liposome volume fraction (β)

Finally, the volume fraction of liposomes in the suspension, β, may be calculated by:

$$\beta = (V_{lv} + V_{hv}) / (V_{lo} + V_{ho}) \quad (8)$$

11. Density of lipid in the aqueous suspension (ρ_l)

The density of the lipids in the aqueous suspension, ρ_l, was evaluated as follows. A known amount of lipids, Q_{lo}, was dispersed in a volume of hemoglobin solution, V_{ho}. The total volume of the suspension was recorded, V_s, and the lipid density was calculated by:

$$\rho_l = Q_{lo} / (V_s - V_{ho}) \quad (9)$$

This treatment was repeated for different concentrations of lipid and the lipid density in the aqueous phase was found equal to 480 g/l of lipids.

Table 1. Symbols and units

[Hb]:	hemoglobin concentration	g/l
Q:	(dry) weight	g
V:	volume	ml or l
α:	aqueous volume fraction in liposomes	(%)
β:	liposome fraction of suspension volume	(%)
η:	fraction of material incorporated into liposomes	(%)
ρ:	specific mass	g/l

Index
- h: hemoglobin
- l: lipids
- s: suspension
- v: in vesicles or liposome
- o: initial

RESULTS

1. Influence of running parameters on encapsulation performances

Three parameters, namely inlet pressure of the microfluidizer, number of recycles, and lipid concentration, have been investigated to evaluate their effect on the encapsulated hemoglobin fraction, η_h, the lipid fraction incorporated in the liposomal membrane, η_l, and the liposomal hemoglobin concentration, $[Hb]_v$.

The formation of liposomes by microfluidizer resulted in high utilization of lipids for membrane formation (η_l up to 85 %) but the hemoglobin encapsulation was limited to 15 % (η_h) (Figures 2 to 4). The aqueous volume fraction, α, inside the liposome was comprised between 20 and 35 % and the liposome hemoglobin concentration, $[Hb]_v$, ranged between 80 and 150 g/l (Figures 2 to 4). By increasing the number of recycles in the microfluidizer (2 to 6), the fraction of lipids incorporated in the liposomal membrane, η_l, is reduced (figure 3). However, the number of recycles does not affect the encapsulation of hemoglobin, η_h, (Figure 2) increasing slightly the liposome hemoglobin concentration, $[Hb]_v$ (Figure 2).

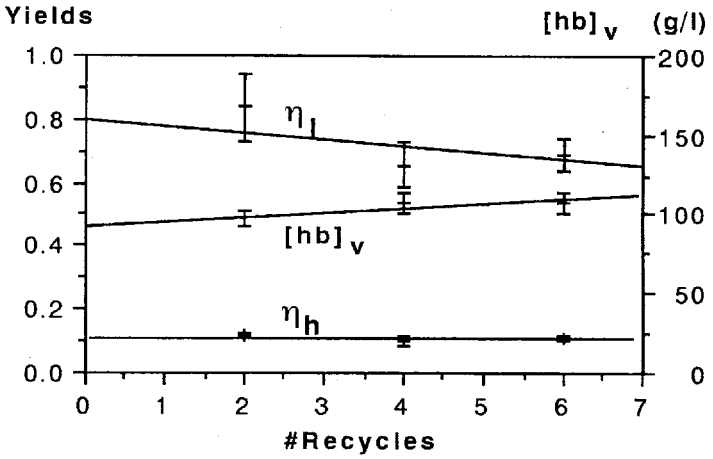

FIGURE 2: Influence of number of recycles on lipid and hemoglobin incorporation yiels,, η_l and η_h, and liposome hemoglobin concentration, $[Hb]_v$

Over the tested range, the inlet pressure had no significant influence on the encapsulated hemoglobin fraction, η_h, and little on the lipid fraction incorporated in the membrane, η_l (Figure 3). The liposome hemoglobin concentration, $[hb]_v$, was only slightly affected by the inlet pressure (Figure 3).

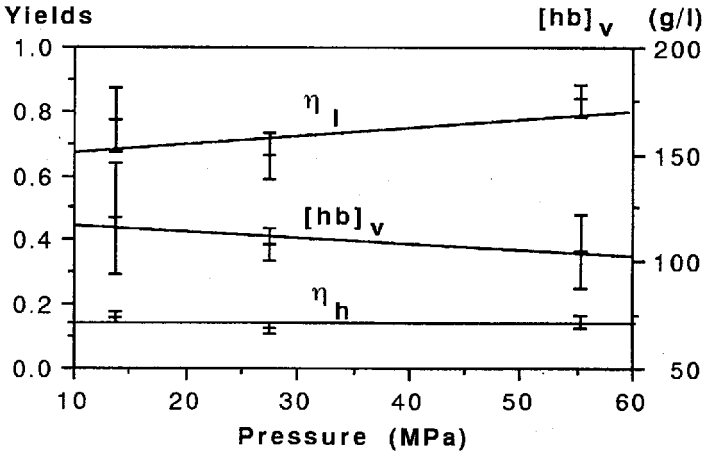

FIGURE 3: Influence of inlet pressure on lipid and hemoglobin incorporation yield, η_l and η_h, and liposome hemoglobin concentration, $[Hb]_v$

Three lipid concentrations were tested for vesicle formation: 120, 240 and 360 g of phospholipid in 1000 ml of hemoglobin solution (subsequently indicated in g/l). However, at phospholipid concentration of 360 g/l (as well as at 240 g/l of phospholipid plus 240 g/l of cholesterol), the viscosity was to high and no liposome could be obtained. Increasing the lipid concentration from 120 to 240 g/l increased the encapsulated

hemoglobin fraction, η_h, (figure 4) from 6 to 10 % and the lipid fraction included in the membrane, η_l, from 51 to 74 %. The mean liposomal hemoglobin concentration, $[Hb]_v$, was higher at lower lipid concentrations (Figure 4).

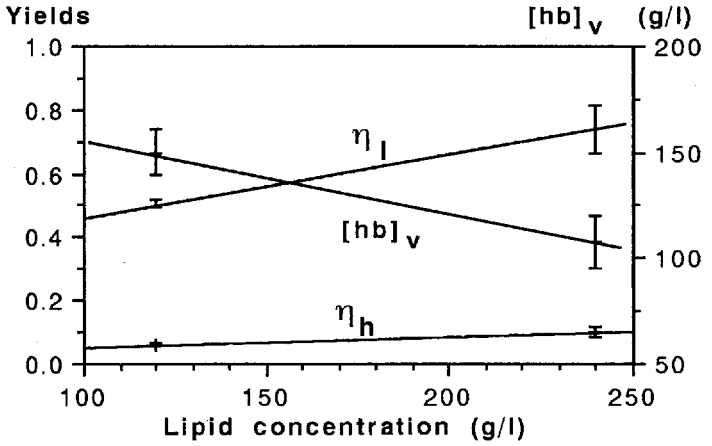

FIGURE 4: Influence of lipid concentration on lipid and hemoglobin incorporation yield, η_l and η_h, and liposome hemoglobin concentration, $[Hb]_v$

Simulations of the encapsulated hemoglobin fraction, η_h, and of the liposome hemoglobin concentration, $[Hb]_v$, are presented in Figures 5 and 6 (dashed lines). These data were computed using different volume fractions of liposomes in suspension, β, and different liposome aqueous fractions, α, and parameters are plotted versus the initial lipid concentration. The lipid incorporation yield, η_l, was assumed to be equal to 100 %. Some experimental results have been reported on this graph after correction of the lipid concentration in function of the lipid incorporation yield, η_l (Figure 5 and 6, solid lines and empty circles).

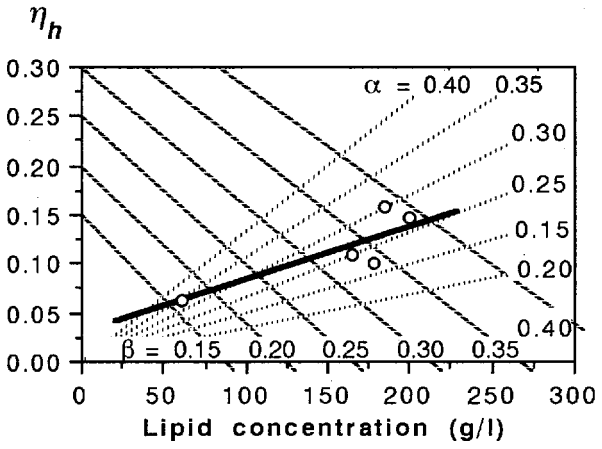

FIGURE 5: Hemoglobin encapsulation yield, η_h, vs the liposome volume fraction, β, or the liposome aqueous fraction, α

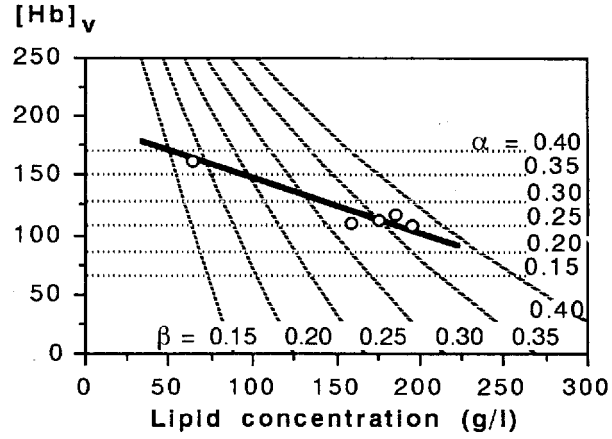

FIGURE 6: Effect of liposome volume fraction, β, and liposome aqueous fraction, α, on the liposome hemoglobin concentration, $[Hb]_v$

2. Liposome shape and stability

Microscopical observations (Figure 7) showed that the microfluidized liposomes were spherical (>75 %). However, some liposomes present irregular forms. Most liposomes were well isolated and very few agglomerates were observed.

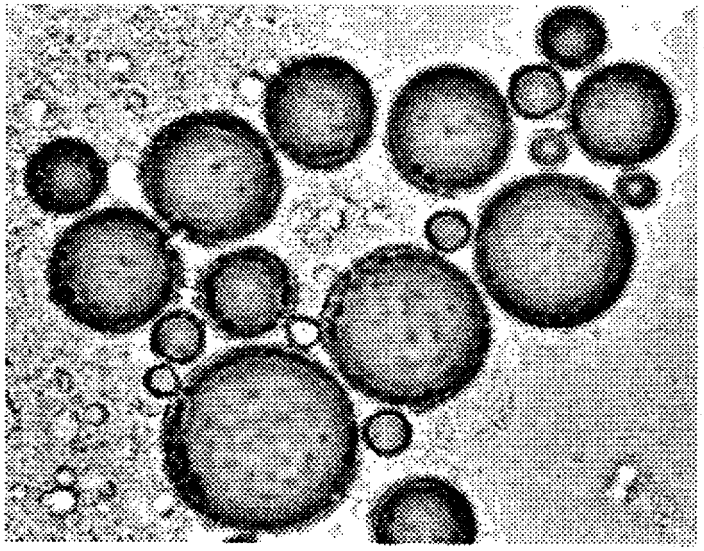

FIGURE 7: Liposomes in the optical microscope (1500X)

Electron microscopy micrographs showed that most liposomes seemed to be intact even after several days of storage (Figure 8). Vesicle stability was also indicated by the fact that the liposomes retained their integrity when suspended in Triton 100X (0.5%). In a few liposomes presenting a broken membrane after fixation, expulsion of hemoglobin content was observed. With electron microscopy, liposome membranes exhibit only a few lamellae.

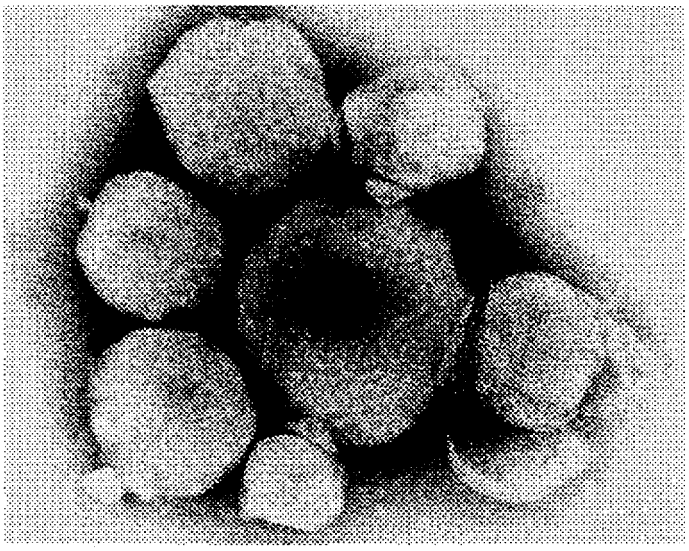

FIGURE 8: Liposomes in the electron microscope (148 500X)

3. Size distribution of liposomes

A typical size distribution of liposomes obtained by microfluidization presents two main peaks (Figure 9). A minor peak is located around 0.5 μm (peak 1) and a major one is around 3 μm (peak 2).

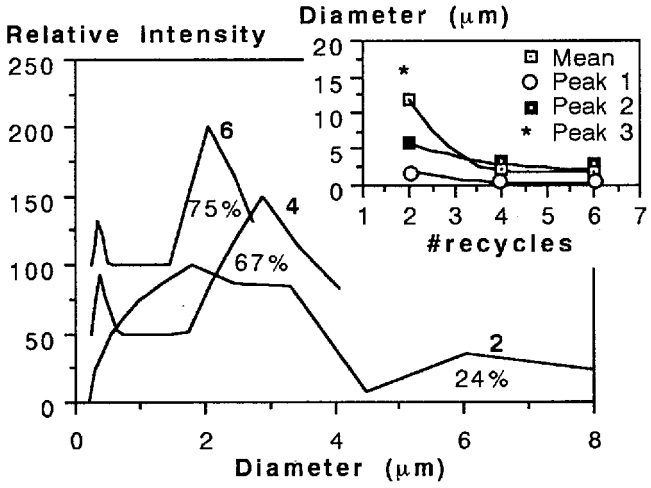

FIGURE 9: Impact of recycle number on liposome size distribution

When only two recycles are applied to the suspension, liposomes present a broad size distribution (Figure 9). The two typical peaks are very large and are not clearly dissociated. Moreover, a third peak appears at around 16 μm (not shown). However, four and six recycles lead to a typical bimodal distribution. The mean height of both peaks decreased reaching a equilibrium value (Figure 9) and the peak 2 relative intensity increased when the number of recycles increased (Figure 9).

Modifications of the inlet pressure do not affect the size distribution, which is still bimodal (Figure 10). When the pressure increased, surface of peak 2 increased (Figure 10) and both peaks shift to lower diameters to reach an equilibrium value (Figure 10). At 55 MPa, peak 2 masks peak 1.

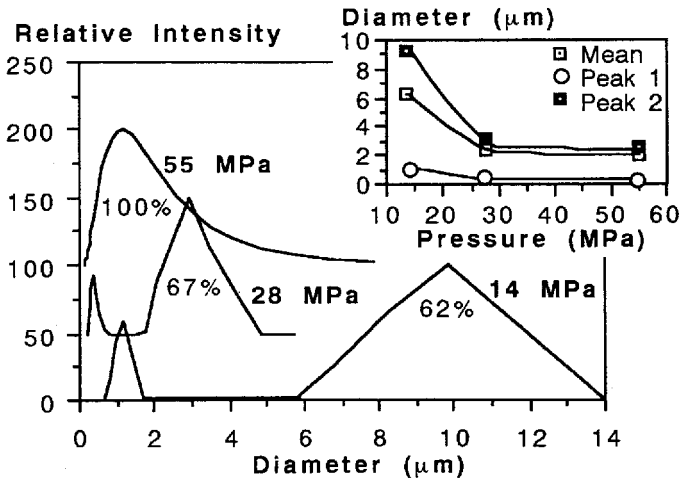

FIGURE 10: Liposome size distribution versus inlet pressure

When the lipid concentration iss doubled (from 120 mg/ml to 240 mg/ml), vesicle size distribution is only slightly displaced towards larger diameters (figure 11).

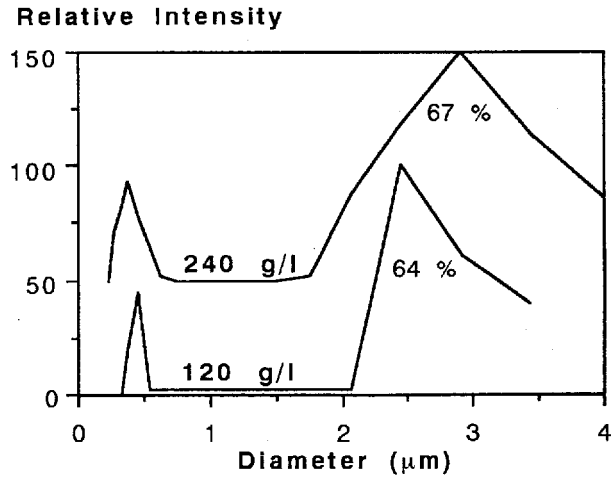

FIGURE 11: Influence of lipid concentration on liposome size distribution

DISCUSSION

A number of methods have been proposed to produce liposomes. The most usual method remains the production of a lipid film on a balloon surface and its disruption with an aqueous solution. Several variants of this method, involving sonication, dehydration-hydration or freeze-thawing, have been proposed to

improve the final properties of vesicles [Hope, 1986; Kirby, 1984b]. However, lack of reproducibility, very low yields of both hemoglobin encapsulation and lipid membrane incorporation, difficulties to scale-up these techniques for industrial use allow to conclude that this process is not realistic from an engineering viewpoint [Vivier, 1991]. Microfluidization of a lipid suspension represents one of the most promising avenues for large-scale liposome production.

In the microfluidization process, a large fraction of lipids is incorporated in the liposomal membrane, η_l (up to 84 %) (Figure 2, 3 and 4). Some liposomes of submicron size may be retained in the supernatant during the centrifugation of liposomes from the hemoglobin solution. The microfluidizer is only preheated, and the temperature of the system then decreased consequently with the number of recycles (Figure 2). At lower lipid concentrations, the lipid yield, η_l, is more sensible to lipid loss (Figure 4). Switching to continuous processing in a temperature control system would result in higher lipid incorporation, η_l, of near to 100 % in an industrial process.

The fraction of encapsulated hemoglobin, η_h, is improved with respect to the lipid film technique (5 %) [Vivier, 1991] and previous results of microfluidization (8 %) [Vidal-Naquet, 1989] but still remains at a low level (up to 15 %) (Figure 2, 3 and 4). The liposome hemoglobin concentration is itself limited to less than 150 g/l. The production of artificial red blood cell is then essentially restricted by the efficiency of the hemoglobin encapsulation.

The liposomes are produced by passing the suspension through very small channels. The suspension must remain fluid to be able to pass through the channels. However, when the volume fraction of vesicles, β, increases the viscosity of the suspension increases, leading to a quasi-solid state. In this solid state, the vesicles are packed and the vesicle volume fraction, β, is around 50 %. In the microfluidization process, the suspension would flow through the channels only if the vesicle fraction, β, remain lower than a maximum value. This maximum is around 35 % (Figures 5 and 6), corresponding to an initial lipid concentration of 240 g/l. At a hemoglobin concentration of 360 g/l, for example, the lipid volume itself represents 42 % of the total volume. Assuming a liposome aqueous volume, α, of 25 %, the liposome volume would be around 55 %.

This limit of the liposome volume fraction, β, results in a limit of the hemoglobin encapsulation yield, η_h. Considering Figure 5, it appears that if the maximum dispersion of the lipid is obtained ($\beta \approx 35$ %), the only way to increase the hemoglobin encapsulation yield, η_h, is to reduce the quantity of lipid incorporated in the liposomes (Figure 5, dashed line, $\beta = 0.35$). However, experimentally, it is observed that reducing the lipid concentration results in a decrease of the liposome volume fraction, β, and then of the hemoglobin encapsulation yield, η_h (Figure 5, solid line) due to a simultaneous reduction of the liposome volume fraction, β. Better efficiency of the dispersion would be needed to obtain a high hemoglobin encapsulation yield, η_h, which is still limited to 15 % with the presently use of microfluidizer.

The liposome hemoglobin concentration, $[Hb]_v$, results mainly from the initial concentration of hemoglobin but also of a diluting factor due to the lipid fraction from the liposome. Hemoglobin-loaded liposomes resulting from microfluidization were still multilamellar with an internal aqueous volume limited to less than 35 % (Figure 6). The liposome hemoglobin concentration remains at a low level (<150 g/l) compared to the red blood cell (≈350 g/l). Reducing the concentration of lipid when maintaining a high liposome volume fraction, β, will lead to high liposome hemoglobin concentration, $[Hb]_v$. This theoretical approach (Figure 6, dashed lines) was partially confirmed by our experimental data (Figure 6, solid line).

From figure 5 and 6, it may be observed that both hemoglobin encapsulation yield, η_h, and liposome hemoglobin concentration, $[Hb]_v$, are essentially controlled by the lipid concentration. The number of recycles and inlet pressure do not affect significantly these value when the lipid concentration is maintained constant (group of points around 175 g/l).

The mean diameter of the liposomes was in the range of 2 to 3 μm, an interesting diameter for a blood substitute, considering that the capillary diameter is around 4 μm [Hunt, 1983]. At least three recycles were needed to obtain an equilibrium for size distribution (Figure 9), the yields and liposome hemoglobin concentration.

Increasing the pressure applied at the entrance of the microfluidizer would increase the flow rate within microfluidizer channels and its disruptive forces. Breakage of vesicles would increase, leading to smaller and more unilamellar liposomes. However, the resulting increase of the liposome volume, ß, would lead to a higher viscosity of the suspension, with, in return, a tendency to lowering the flow rate again. The pressure effects are then buffering. Moreover, the resistance of the vesicle to breakage is strongly related to vesicle size. Decreasing size leads to stronger vesicles [Poncelet, 1989]. Pressure increases will be less and less effective. Looking at these two effects, it is not surprising that yields were not strongly affected by pressure changes (Figure 2) and that the mean liposome diameter reaches a equilibrium value when pressure is increased (Figure 10). Improving the encapsulation efficiency implies a better and more homogeneous use of energy in the breakage zone than an increase of energy dissipation.

Microfluidization remains one of the most interesting processes to produce hemoglobin-containing liposomes on a large scale. Some improvements are still needed to reach the objectives. The batch process must be converted to a continuous process. Direct control of the flow rate in place of the pressure and a non pulsed flow would allow a better control of dispersive forces. A better knowledge of the breakage process in the interaction chambers is necessary. However, the present results show that the key is probably in the handling of lipid. Lower lipid concentrations must be used. By increasing the temperature, the lipid membrane would become more fluid and then more amenable to opening and rebuilding. To avoid hemoglobin denaturation, it will be necessary to work in strict anaerobic conditions, increasing the temperature only during very short time needed to process the suspension.

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