International Journal of Pharmaceutics, 24 (1985) 149–164
Elsevier

IJP 00814

Vinblastine sulphate encapsulation in phospholipid vesicles and liposomes

Lisbeth Ter-Minassian-Saraga and Geneviève Albrecht

Physico-Chimie des Surfaces et des Membranes, Equipe de Recherche du CNRS associée à l'Université Paris V, UER Biomédicale, 75270 Paris Cedex 06 (France)

(Received July 16th, 1984)
(Accepted November 30th, 1984)

Summary

Vinblastine sulphate (VBLS) encapsulation by small phospholipid (mainly egg lecithin (EL)) vesicles and by large egg lecithin liposomes was studied. It is found that liposomes encapsulate 10 times more drug (~ 2% mol fraction compared to ~ 2‰ mol fraction) than the small vesicles (60–80 nm diameter). The encapsulation of VBLS may be increased by a factor of 5 when the ab initio (in the dry lipid film) ratio VBLS/EL varies from 0.03 to 1. It is assumed that this increase is due to entrapment of segregated drug in or between the EL bilayers. The release of the entrapped drug into fresh buffer is a slow phenomenon.

Introduction

Vinblastine sulphate (VBLS) and its derivative vincristine sulphate (VCRS), amphiphilic dimeric alkaloids used in cancer chemotherapy (Owellen et al., 1977; Nimni, 1972), have a limited utility on account of their neurological toxicity. To reduce it, experiments with VBLS and VCRS encapsulated in small sonicated liposomes have been carried out (Juliano and Stamp, 1978; Layton et al., 1979; Layton and Trouet, 1980). It has been found (Layton and Trouet, 1980) that the VCRS doses used involved the delivery of toxic amounts of phospholipids.

In this article we show that vesicle VBLS encapsulation is low, as reported by Layton et al. (1979) and Layton and Trouet (1980), and that the level and the stability of encapsulated VBLS may be raised in large phospholipid liposomes only, by increasing the ab initio ratio VBLS/phospholipid in the system.

Correspondence: L. Ter-Minassian-Saraga, c/o Professor T. Takenaka, Institute for Chemical Research, Kyoto University, Uji, Kyoto-Fu 611, Japan.

It is speculated that this cooperative-like enhancement of VBLS encapsulation in liposomes may be related to a bilayer phase separation at a given (encapsulated) VBLS concentration.

Materials and Methods

Materials

Egg lecithin (EL) and phosphatidylinositol (PI) were obtained from Lipid Products (U.K.). Samples corresponding to various batches produced similar liposomes and vesicles.

To prevent EL hydrolysis on storage in $CHCl_3/CH_3OH$ solution, on receiving the samples they were dried out under reduced pressure and either redissolved in cyclohexane under N_2 or kept dry under N_2. They were stored at $-18°C$. HPC chromatograms of 6 μl phospholipids solution (100 mg/ml) revealed the following acyl compositions: 56% C-(16 : 0), 7.5% C-(18 : 0), 26% C-(18 : 1), 10% C-(18 : 2) and 69% C-(16 : 0), less than 1% C-(18 : 1), ~ 30% C-(18 : 2) for EL and PI, respectively.

VBLS was a gift from Eli-Lilly. Cholesterol was Merck grade. The ^3H-labelled vinblastine sulphate (9.9 C/mmol^{-1}, radiochemical purity 95–97%) was purchased from Amersham (U.K.) and was used not later than 3 months after reception. The solvents CH_3OH, $CHCl_3$ were Merck analytical grade. Cyclohexane was Merck spectroscopic grade. Na cholate grade Merck (> 99%), the solvents, VBLS and EL were used as received. The phosphate buffer was prepared from NaCl, NaOH, NaH_2PO_4 analytical grade and tridistilled (once on $KMnO_4$) water. The mother solutions of the salts were purified by prolonged foaming (with bubbling N_2) and sucking the foam off the salt solution surface until exhaustion of persistent foam.

Methods

Vesicle preparation and characterization. The vesicles were prepared by the fast and controlled dialysis of mixed lipid–detergent micelles (Milsmann et al., 1978). The triple-cell dialysis apparatus Lipoprep-Dianorm (Diachem A.G.) and the detergent sodium cholate were used. The mixed lipid + VBLS dry film was formed from solutions in $CHCl_3/CH_3OH$ (9/1) by rotary evaporation at room temperature. The ratios lipid/drug or, eventually (lipid + cholesterol)/drug were varied. The dry film was dispersed mechanically by shaking with 6–10 ml of a 1 mM phosphate, 0.16 M NaCl and 0.2% sodium azide buffer (pH 7.3). Sodium cholate was then added to obtain a lipid/detergent 0.6/1 (mol/mol) composition. The dispersion was shaken until clear and left overnight at 4°C. The final dispersion contained 13–20 mg lipid/ml. Its controlled dialysis was carried out at 15–20°C for 24 h at a stirring rate 75 min^{-1} and a flow rate of buffer 3 ml · min^{-1}. Under these conditions (see Table 2; Milsmann et al., 1978) the residual cholate in the vesicles is less than 0.5% (mol/mol). The vesicle dispersion was assayed for total P using the Serva kits based on the method of Eibl and Lands (1969). We have calibrated this method with known amounts of DPPC and found out that 30 μl of H_2O_2 and incubation for 30 min at 30°C were necessary for accurate ($\simeq$ 100%) assay of the lipid. A control assay

of the buffer P contribution was carried out too. The difference between total P and buffer P provided the lipid P. In our case the P-lipid/P-buffer was 8/1 (mol/mol). The optical density was measured with Kontron spectrophotometer (U.K.). The accuracy of P-lipid assay was 5% (mol/mol).

The VBLS was assayed using a scintillation cocktail Picofluor 30 (France) and a scintillation counter Packard 3002 which included computerized background subtraction. Its calibration was carried out using both aliquots of the mother [^{3}H] VBLS solution and the vesicle dispersion right after its dialysis. The counting time was 4 min.

The vesicles were separated from the external (free) VBLS by gel filtration (0.5 ml or 1 ml samples) on pre-equilibrated Sepharose 4B columns (0.7 ml injection) and the partition coefficient K_{app} of VBLS between vesicles and external solution was obtained from the areas of peaks corresponding to VBLS—liposome bound or free VBLS in the elution patterns. The total amount of VBLS and EL recovered after gel filtration on Sepharose 4B was $85 \pm 10\%$ and $90 \pm 5\%$, respectively (average of 13 experiments). A similar result has been reported by Layton et al. (1979).

The vesicles' size was determined by gel filtration on Sephacryl S-1000 following Nozaki et al. (1982). These results were occasionally checked by determining the vesicle hydrodynamic radius from studies of (vesicle) apparent diffusion constant using the light scattering method, an argon laser (300 mW) and a Maldern correlator. These experiments were carried out at 10°C and 20°C. A 2–10% polydispersity of the various vesicle dispersions was thus found.

The internal volume and the associated VBLS were determined. The vesicles were eluted with NaCl 0.16 M to remove the external buffer P. Ultrafiltration by suction through a collodium filter (75,000 M_w retention) concentrated the eluted vesicles dispersion to about 15% (lipid/H_2O, w/w) using a LMR-COLL (Labo Moderne, France) device and the NaCl 0.16 M filtrate was assayed for [^{3}H]VBLS to detect vesicle leakage. Then, the vesicles were incubated with water at 4°C for 24 h to break them (osmotic shock) and liberate the encapsulated buffer P. The solution was removed by ultra-filtration and assayed for mineral P and VBLS. Assuming that the internal phosphate concentration was 10^{-3} M (the buffer concentration) the internal vesicle volume was obtained by dividing the mineral P found by 10^{-3} M.

Experiments of penetration (partition and kinetics) of VBLS into "empty" lipid vesicles were carried out by incubating them with buffer solutions of [^{3}H]VBLS at 4°C for various periods of time followed by gel filtration on Sepharose 4B and/or Sephacryl S-1000. Some EL vesicles, turned into EL liposomes irreversibly after 24 h storage at −19°C, were incubated with VBLS solutions up to 7 days. For these experiments the EL and VBLS concentrations were 1.6×10^{-2} mmol · ml^{-1} and 6.2×10^{-4} M, respectively. The vesicle or liposome dispersion volume was in the range 2–5 ml. It was constant in a given series of experiments.

Liposome preparation and characterization. A dry film of EL + [^{3}H]VBLS was prepared in plastic tubes and dispersed by shaking with 5 ml phosphate buffer 10^{-3} M and 0.16 M NaCl to obtain a lipid concentration of 0.3–1.5 mg/ml. The ab initio ratio lipid/drug was varied in the range 34/1 to 1/1 (EL/VBLS, mol/mol). The rough 5 ml dispersion is sonicated for 1 min in a Bransonic 50 kcycles bath sonicator

at room temperature and left overnight (~ 17 h) at 4°C. It is then resonicated for 1 min and duplicate samples 50–200 μl are assayed for VBLS and lipid P as described above. It was assumed that, during the night, the adsorption on the tube walls was complete and that the "overall" VBLS concentration thus determined was corrected for adsorption on the tube wall. The dispersion was then centrifuged at $3000 \times g$ for 30 min and 0.5 ml of supernatant (duplicate sample) was assayed for VBLS. The difference between the overall VBLS concentration and that of supernatant provides the concentration of encapsulated drug $(VBLS/EL)_{enc}$ (mol/mol). In these experiments, as well as in the subsequent rinsing experiments (see below) the centrifugation pellet volume was negligible (< 1%) compared to the total volume equal to either 2 ml or 5 ml. While the drug/lipid ab initio mol ratio (i.e. in the dry lipid + VBLS film) varied in the range 0.03 (mol/mol)–1 (mol/mol), EL concentration was in the range 4×10^{-4} M–2×10^{-2} M and the overall VBLS concentration changed within the limits 6×10^{-4} M–4×10^{-3} M. The ab initio mol fraction of VBLS in these systems is x = [VBLS/(VBLS + EL), mol/mol] where VBLS, EL are the ab initio total numbers of moles in the dispersion. In our experiments x varied between ~ 0.03 and 0.5.

To study leakage (or desorption) of VBLS from liposomes, practically all supernatant was removed carefully, replaced by 5 ml of fresh buffer and the pellet was redispersed by vigorous shaking. Contact was allowed for 24 h at 4°C before the second centrifugation and supernatant assay for VBLS were carried out. This "rinsing" process was repeated twice. The pellet of the fourth centrifugation was redispersed by 1 min sonication with 1 ml fresh buffer, assayed for lipid-P and the, eventual, loss of lipid during the rinsing experiments was estimated. On the average of 10 experiments, 85 ± 10% of the initial EL was present. The VBLS concentration $(VBLS/EL)_{enc}$ (mol/mol) and the partition coefficient $K_{app} = [VBLS/ELc_{VBLS}]$ $[l \cdot mol^{-1}]$ were deduced. $[c_{VBLS}]$ is the concentration of free VBLS in the supernatant. $(VBLS/EL)_{enc}$ and K_{app} were deduced assuming that all the lipid was present. These results were at most 15% too low.

The encapsulation of VBLS by incubating pure EL liposomes with VBLS solutions and the stability of the encapsulated drug on rinsing were also studied. These series of experiments were performed using the same conditions, i.e. total volumes and drug/EL ratios in the system as for the ab initio VBLS-incorporated experiments described above. In this case, VBLS concentrations in the incubating solutions were in the range 6×10^{-4} M–4×10^{-3} M also.

All the incubations and "rinsing" experiments were carried out at 4°C.

In one case [ratio (VBLS/EL) = 0.03 mol/mol], the incubation was studied for 1–7 day contact periods. As no effect of time on the encapsulated drug concentration was observed, all the experiments of incubation and rinsing were carried out by allowing contact periods of at least 24 h at 4°C. In this experiment too the drug and EL concentration were 6.2×10^{-4} M and 2×10^{-2} M, respectively.

The internal volume of the liposomes was measured by assaying the encapsulated mineral P. To favour complete liberation of the mineral P, the liposomes were incubated with water at 4°C in the presence of chloroform (5/1, $EL/CHCl_3$, mol/mol).

Results

Vesicle characterization and kinetics of VBLS encapsulation

Fig. 1a and b represent the elution patterns of VBLS for PC and PC/PI/Ch vesicles, respectively, after 24 h dialysis, or time 0, and after 9 days storage at 4°C. Peaks 1 and 2 corresponded, respectively, to the encapsulated and free drug. Their areas or respective amounts of VBLS displayed a small time evolution (see Table 1, column 5).

The percentage of VBLS encapsulated was not reproducible: 0.6–10% (of original drug). The concentration of free VBLS increased with the initial VBLS/lipid ratio. The elution pattern of EL (Fig. 1a) was studied. The total amounts of EL associated with the peaks 1 and 2 of the VBLS elution pattern have been determined (Fig. 1a

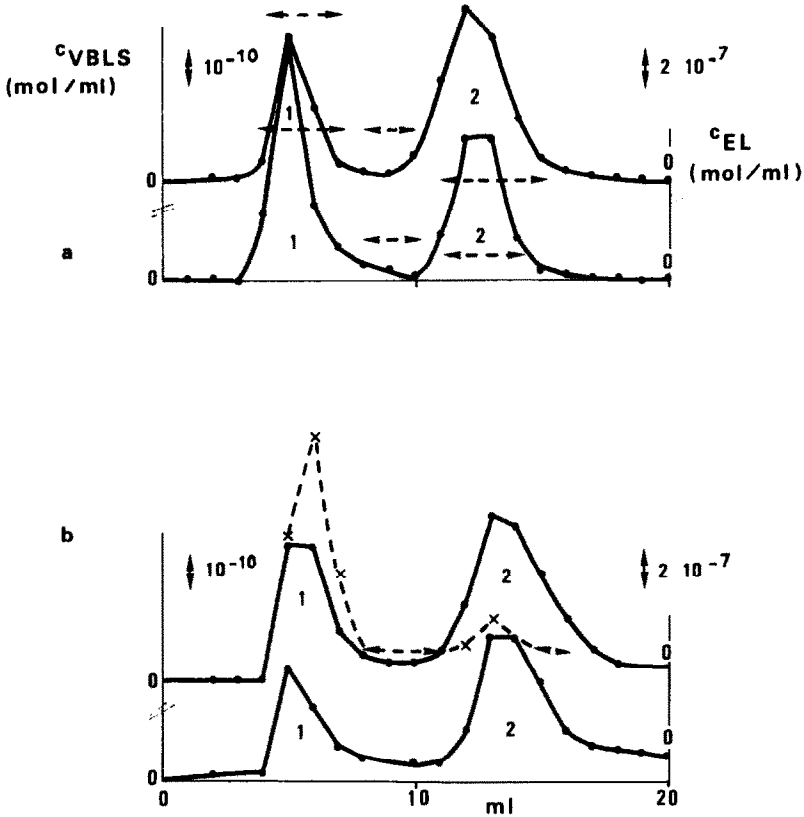

Fig. 1. Elution patterns of VBLS-loaded vesicles at 4°C. (a) PC vesicles; (b) PC/PI/Ch, 5/2/5 (mol/mol) vesicles. Sepharose 4B column. Injected volume 0.7 ml; 1 ml samples. VBLS pattern—full line. Peak 1: encapsulated VBLS; Peak 2: free VBLS. c_{VBLS}, VBLS concentration in samples. c_{EL}, EL concentration in samples. Scales, respectively, 10^{-10} mol/ml, 2×10^{-7} mol/ml. (a) or (b): upper pattern after vesicle preparation; lower pattern: after 9 days storage at 4°C. (a) dashed arrows: volume assayed for EL; (b) dashed chromatogram: EL.

TABLE 1

VBLS ENCAPSULATION BY PHOSPHOLIPID VESICLES

System (mol/mol)	Lipid P (M)	$(\frac{VBLS}{P})_{enc}$ (mol/mol)	$(VBLS)_{free}$ [M]	K_{app} (l/mol)	Time (days)	% $(VBLS)_{enc}$
EL/PI/Ch (5/2/5)	1.09×10^{-2}	0.0030	1.7×10^{-5}	150	5	10
	1.09×10^{-2}	0.0030	1.7×10^{-5}	150	22	10
EL/PI/Ch (5/2/5)	1.2×10^{-2}	0.0007	1.6×10^{-5}	42	0	2.6
	1.2×10^{-2}	0.0007	1.6×10^{-5}	42	9	2.6
EL/PI/Ch (5/2/5)	8.3×10^{-3}	0.0004	5.6×10^{-6}	66	0	1.3
	8.3×10^{-3}	0.0003	6.3×10^{-6}	48	9	1.0
EL	6.7×10^{-3}	0.001	1.7×10^{-5}	65	0	3.3
	6.7×10^{-3}	0.002	1.3×10^{-5}	134	9	7.0
EL*	8.5×10^{-3}	0.0015	1.75×10^{-4}	~ 9	0	0.6
		0.0015	1.75×10^{-4}	~ 9	9	0.6

Encapsulation by fast controlled dialysis. $(VBLS/P)_d$ = ab initio ratio (in the dry film): 0.03 (mol/mol) and 0.25* (mol/mol). Time = days after end of dialysis. $(VBLS/P)_{enc}$ = ratio for the eluted vesicles (Peak 1, Fig. 1a and b). $(VBLS)_{free}$ = VBLS concentration of the eluted intervesicle solution (Peak 2, Fig. 1a and b). K_{app} = apparent partition coefficient (see text). % $(VBLS)_{enc} = [100\times(VBLS/P)_{enc}]/(VBLS/P)_d$.

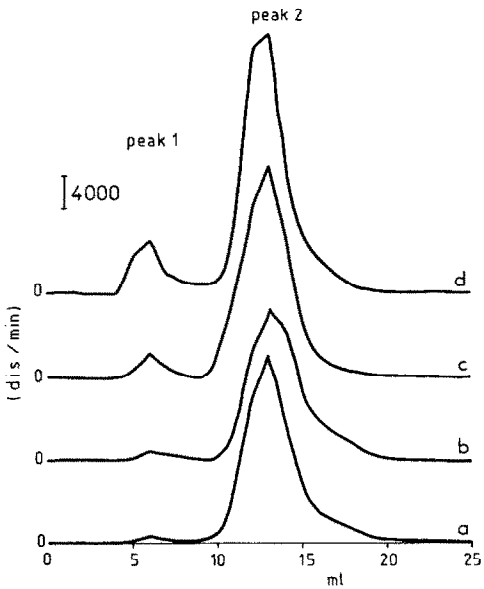

Fig. 2. Elution patterns of EL vesicles incubated with VBLS at 4°C. $c_{VBLS} = 6.2\times10^{-4}$ M; $c_{EL} = 1.6\times10^{-2}$ M (see also legend of Fig. 1). VBLS concentration in samples in dpm. For scale see arrow. Duration of incubation: a, 6 h; b, 3 days; c, 6 days; d, 14 days.

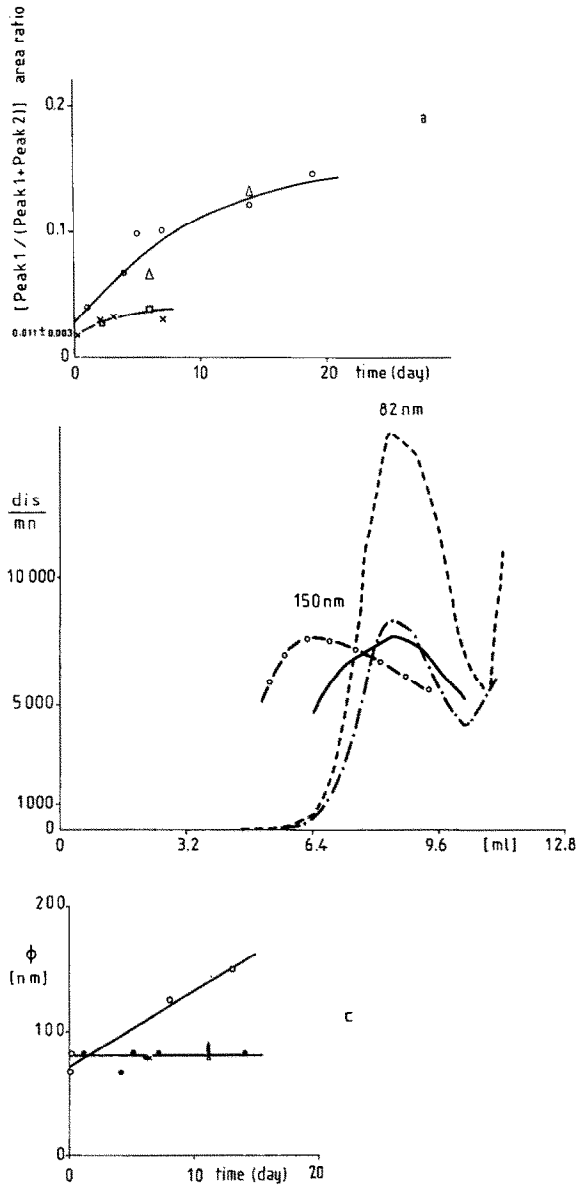

Fig. 3. Time evolution of VBLS encapsulation and EL vesicle size at 4°C. (a) % of encapsulated VBLS obtained from peaks' areas of patterns in Fig. 2. vs duration of incubation. Results of experiments with four EL vesicle batches (×, □, △, ○) (see also legends of Figs. 1 and 2). Sepharose 4B columns. (b) Same as (a) above. Sephacryl S-1000 column (void volume 37%) and 0.64 ml samples. Only vesicle peak 1 is shown. Control (unloaded vesicles) at origin of experiment (full line) and 13 days later (○–○). Incubated vesicles at origin of experiment (·–·–·) and 14 days later (- - - - - -). Numbers correspond to vesicle diameter determined according to Nozaki et al. (1982). (c) Time evolution of vesicle diameter (⌀) at 4°C, ○, ● from elution patterns (see b), respectively, for control and VBLS-loaded EL vesicles. △: by light scattering (photon correlation spectrometry).

and b). The values $(VBLS/P)_{enc}$ obtained for the encapsulated drug are shown in Table 1. No significant lipid-P has been located at peak 2. The total amount of vesicle lipid-P recovered corresponded to $90 \pm 5\%$ of the initial amount of EL. This amount of EL recovery was satisfactory in contrast to the percentage of drug encapsulation. A factor of 10 in the free VBLS concentration (Table 1) did not significantly affect the efficiency of VBLS encapsulation.

The results of VBLS encapsulation experiments carried out by incubating at 4°C EL vesicles with VBLS solutions are shown in Figs. 2 and 3.

To a dispersion of vesicles of EL 1.6×10^{-2} M, ^{3}H-labelled VBLS was added. The VBLS assay provided the overall concentration 6.2×10^{-4} M. The incubation at 4°C was carried out for various time periods. Several incubations were carried out at 20°C. The bound VBLS was separated from the free form by gel filtration. The elution patterns in Fig. 2 correspond to incubation periods of 6 h to 14 days. The ratio of encapsulated (peak 1) to free VBLS (peak 2) increases with the incubation period. The increase of the percentage of encapsulated VBLS with time is shown in Fig. 3a where we plotted the fraction of peak 1 area of the total, peak 1 and peak 2 areas, measured on elution patterns of VBLS (Fig. 2). The results in Fig. 3a have been obtained with four different batches of EL vesicles. The percentage of immediately ($t = 0$) encapsulated drug was $1.1 \pm 0.3\%$ corresponding to a calculated mol fraction of VBLS equal to 0.0008 ± 0.0003. The temperature (4°C or 20°C) effect was less significant (Fig. 3a) considering the reproducibility of these results. However, both lines in Fig. 3a indicate a saturation for VBLS encapsulation.

The increase of VBLS encapsulation with the incubation period was not related to an evolution of vesicle size as shown by Fig. 3b and c. The loaded vesicles' diameter, measured by gel filtration, was time-independent and about 80 nm for both preparations studied. The triangles in Fig. 3c correspond to the hydrodynamic diameter determined by light scattering. For the same period of time the control (without VBLS) EL vesicles diameter almost doubled (Fig. 3b and c). The stability (towards fusion) of the drug-carrying vesicles is probably related to the presence of VBLS molecules. The chemical instability of the drug did not allow us to continue the incubation experiments beyond 19 days.

In the course of the incubation experiment 1 (Fig. 3a), from $t = 1$ day to $t = 14$ days, the VBLS encapsulation increased from 1% to 14%, i.e. by about 13%. The variation in 6 days of VBLS encapsulation in experiment 2 was about 2.0%.

The vesicle internal volume was determined from the amount of encapsulated buffer phosphate (10^{-3} M) as explained under Methods. Experiments were carried out (see Table 2) with two vesicle preparations. The measured encapsulating volumes were quite reproducible: 0.5% of 0.40% (v/v) for v_v and 2 l/mol EL and 1.2 l/mol EL for v_L, where v_v and v_L are the internal volumes reduced either to 1 ml of dispersion or to 1 mol EL, respectively.

Fig. 4 represents a vesicle of hydrodynamic diameter of 80 nm, the measured value. The "wall" thickness, equal to 6.2 nm, corresponds to the repeat distance reported by Parsegian et al. (1979), i.e. to a lipid core 3.5 nm plus two half structural water layers 1.35 nm thick each. The internal aqueous volume includes the internal structural water layer H_2O_{str}. Using the EL (vesicle) concentrations (column 1, Table

TABLE 2

EVALUATION OF VESICLE ENCAPSULATED (INTERNAL) VOLUME. VBLS INCUBATED VESICLES

EL ($\frac{mmol}{ml}$)	Phosphate P ($\frac{mmol}{ml}$)	v_{total} (ml)	v_L (l/mol)		v_v% (v/v)	
			calc.	obs.	calc.	obs.
2.7×10^{-3}	5.3×10^{-6}	3.7	2.4	2.0	0.66	0.53
3.05×10^{-3}	3.6×10^{-6}	13.0	2.4	1.2	0.75	0.36

Concentration of encapsulated buffer phosphate 10^{-3} M. EL = determined by lipid P assay in the eluted vesicle dispersion of total volume, v_{total}. P = mineral (buffer) phosphate assayed in the H_2O solution after osmotic shock. v_L, v_v as defined in text.

2) and those of mineral (buffer) phosphate (column 2, Table 2) the reduced volumes v_v and v_L have been estimated (columns 4 and 5, Table 2). The discrepancy between the estimated and the observed values may originate in the loss of some phosphate during the experiment (adsorption or insufficient rinsing). However, an order of magnitude agreement between the measured values of R_H by gel filtration and light scattering, on one hand, and the value deduced from internal volume estimation, on the other hand, was obtained (Table 2).

We may compare the result for the vesicle internal volume with the results of VBLS incubation experiments. If no binding of VBLS to vesicles wall occurred, the encapsulation of VBLS inside the vesicle would have involved a maximum depletion of VBLS in the presence of EL vesicles of about 0.4% to 0.5%. At time t = 0 a depletion of about 1.1% was measured. It increased with time to 14%.

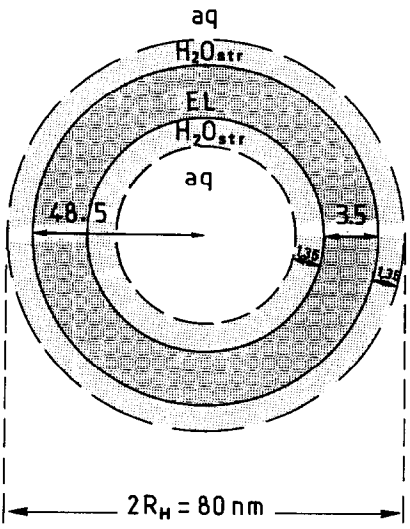

Fig. 4. EL vesicle (not to scale). R_H = hydrodynamic radius ($\varnothing/2$) (see Fig. 3c). H_2O_{str} = layer of structural water = 1.35 nm, bilayer thickness = 3.5 nm.

TABLE 3

VBLS PARTITION BETWEEN EL LIPOSOMES AND SUPERNATANT. EFFECT OF DILUTION
(A) AB INITIO INCORPORATED VBLS

	Dilution	0			1			2		
x		c_{VBLS} (M)	$(\frac{VBLS}{EL})_{enc}$ (mol/mol)	K_{app} ($l \cdot mol^{-1}$)	c_{VBLS} (M)	$(\frac{VBLS}{EL})_{enc}$ (mol/mol)	K_{app} ($l \cdot mol^{-1}$)	c_{VBLS} (M)	$(\frac{VBLS}{EL})_{enc}$ (mol/mol)	K_{app} ($l \cdot mol^{-1}$)
0.5		3.4×10^{-4}	0.125	368	1.1×10^{-5}	0.100	9000	2×10^{-6}	0.09	45000
		3.7×10^{-4}	0.100	275	1.6×10^{-5}	0.053	3300	2.4×10^{-6}	0.056	19200
average				319			6150			32100
0.33		1.9×10^{-4}	0.051	270	7.6×10^{-6}	0.029	3800	3.6×10^{-6}	0.018	5000
0.25		2.1×10^{-4}	0.071	338	3.8×10^{-5}	0.051	1340	1.2×10^{-5}	0.045	3750
		1.2×10^{-4}	0.050	416	5.6×10^{-6}	0.033	5900	3.2×10^{-6}	0.024	7500
average				377			3620			5650
0.2		1.6×10^{-4}	0.050	308	3.2×10^{-5}	0.034	1060	1.1×10^{-5}	0.028	2518
		9.5×10^{-5}	0.030	315	4.7×10^{-6}	0.016	3400	3×10^{-6}	0.0066	2200
		8.9×10^{-5}	0.026	292	5.4×10^{-6}	0.010	1850	2×10^{-6}	0.0034	1700
average				305			2100			1950
0.03		9.7×10^{-5}	0.020	206	3.1×10^{-5}	0.017	550	2×10^{-5}	0.015	750
		1.1×10^{-4}	0.019	173	3.3×10^{-5}	0.015	450	2×10^{-5}	0.013	650
		8.5×10^{-5}	0.021	247		0.019			0.016	
average				209			500			700

(B) LIPOSOMES INCUBATED WITH VBLS SOLUTIONS

x	Dilution 0			1			2		
	c_{VBLS} (M)	$(\frac{VBLS}{EL})_{enc}$ (mol/mol)	K_{app} ($l \cdot mol^{-1}$)	c_{VBLS} (M)	$(\frac{VBLS}{EL})_{enc}$ (mol/mol)	K_{app} ($l \cdot mol^{-1}$)	c_{VBLS} (M)	$(\frac{VBLS}{EL})_{enc}$ (mol/mol)	K_{app} ($l \cdot mol^{-1}$)
0.25	2.2×10^{-4}	0.057	260	2.6×10^{-5}	0.045	1730	6.9×10^{-6}	0.041	5900
0.20	1.8×10^{-4}	0.026	144	2.3×10^{-5}	0.014	610	6.6×10^{-6}	0.011	1650
0.03	3.8×10^{-4}	0.011	29	1.0×10^{-4}	0.007	70	5.4×10^{-5}	0.0046	85
0.03	3.0×10^{-4}	0.014	47	1.0×10^{-4}	0.010	100	7.1×10^{-5}	0.0061	86

x = VBLS mole fraction in dry VBLS + EL film. c_{VBLS} = VBLS concentration in supernatant. $(VBLS/EL)_{enc}$ = ratio for the pellet; $K_{app} = [(VBLS/EL)_{enc}/c_{VBLS}]$. Dilution: the pellet of dilution 0 was redispersed in fresh buffer 1, 2, 3 times and centrifuged (see Methods). Results for dilution 3 not reported.

Encapsulation and desorption experiments with EL liposomes and VBLS

The results of VBLS encapsulation in EL liposomes are shown in Tables 3a, 3b and Table 4 and Figs. 5a and b and Fig. 6.

The lines in Fig. 5a show the effect of the ab initio composition of the dry VBLS + EL film on the compositions of the liposomes after their sonication with buffer and centrifugation $(VBLS/EL)_{enc}$(dilution 0, Table 3a) and on the equilibrium concentration of the free VBLS, c_{VBLS}. Each line corresponds to one ab initio mol fraction x = [mol VBLS/(mol VBLS + mol EL)] of VBLS. In Fig. 5b we show the variation of this equilibrium concentration c_{VBLS} with x. The results in Fig. 5a and Fig. 5b, for $0.2 < x < 0.5$ correspond to an average constant value of $K_{app} \simeq 320\ (l \cdot mol^{-1})$ for these initial compositions while for x = 0.03 a lower value

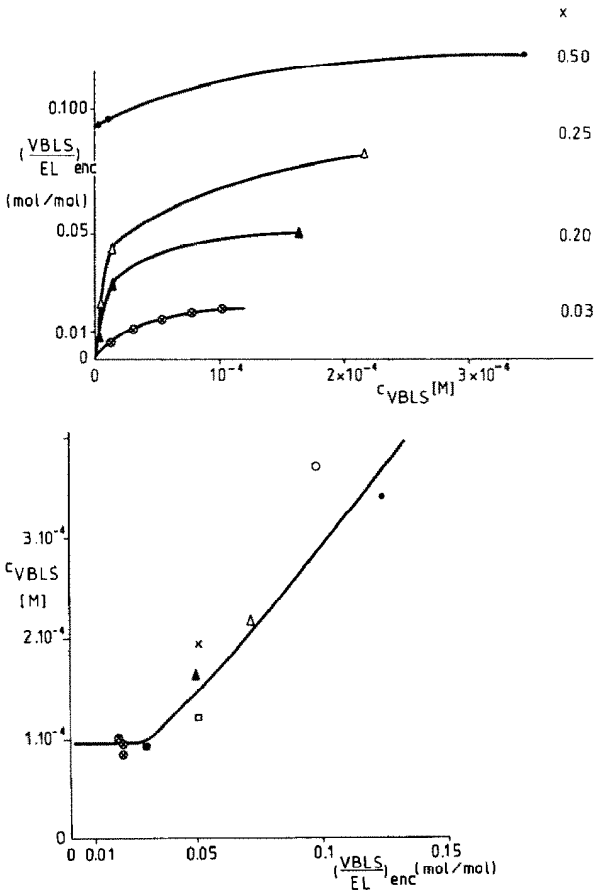

Fig. 5. Variation of VBLS encapsulation in EL liposomes with x the overall mole fraction of VBLS at 4°C. x = [VBLS/(VBLS + EL)] (mol/mol). c_{VBLS}, supernatant concentration; $(VBLS/EL)_{enc}$ (mol/mol), VBLS concentration in the liposome pellet. (a) Compositions of supernatant and pellet (see also Table 3a). (b) Partition of VBLS between liposome and supernatant (based on results of a). ○● (x = 0.5); × (x = 0.33); △□ (x = 0.25); ▲■ (x = 0.2); ⊗ (x = 0.03).

$K_{app} \simeq 210$ ($l \cdot mol^{-1}$) is obtained (see Table 3a). Therefore at $x \simeq 0.25$ or VBLS/EL $= (1/3)$(mol/mol) the mechanism of VBLS retention by the EL liposomes has changed. The effect of the ab initio composition x on VBLS encapsulation equilibrium and stability is amplified at the lower concentrations of free VBLS, c_{VBLS}, involved by the successive dilutions of the centrifugation pellets. Thus while the supernatant c_{VBLS} decreases (Fig. 5a, Table 3a), K_{app} raises and the rate of K_{app} increase with supernatant dilution, increases with x, the ab initio mol fraction of VBLS. Thus at dilution 2 (Table 3a), when the order of magnitude of the physiological concentration $c_{VBLS} \simeq 10^{-6}$ M is attained, K_{app} for $x = 0.5$ was 16 times larger than that for $x = 0.2$, while for c_{VBLS} of the order of 10^{-4} M (dilution 0) no significant difference in the corresponding values of K_{app} was noticed. Therefore an ab initio high concentration of VBLS stabilizes the encapsulated VBLS, on the one hand, and enhances the efficiency of the drug encapsulation on the other. While the VBLS concentration in vesicle is about 0.1–0.2 mol% for $x = 0.03$ (Table 1, last 4 rows), under similar conditions, the VBLS concentration in liposomes is 10 times more: 1.4–2.0 mol%.

For $x = 0.03$ the internal volume was determined by assaying the mineral P encapsulated. A negative result was obtained, implying either a leaky structure or a too small internal volume of the liposomes. Therefore all the retained (by the liposomes) VBLS was strongly bound to EL multibilayers.

Experiments of incubation at 4°C of EL liposomes with VBLS solutions and subsequent dilutions with buffer were carried out. The results are shown in Fig. 6, Table 3b and Table 4. For these experiments x represents the ab initio VBLS mol fraction *in the system* EL liposome + VBLS solution. Only the "compositions" $x = 0.03$, 0.2 and 0.25 mol% were studied. The experimental conditions (amounts of substances and buffer) were identical to those for the experiments reported above (Table 3a). The results obtained (Table 3b) were qualitatively similar to those reported in Table 3a. Increasing the mole fraction x (in the system) and diluting the supernatant increased the partition coefficient K_{app} and the drug encapsulation (VBLS/EL) (mol/mol) (Table 3b). The similarity of drug encapsulation and of its

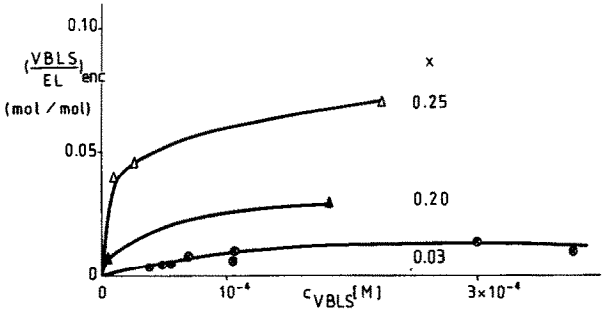

Fig. 6. Encapsulation of VBLS by incubation of EL liposomes with VBLS solutions and stability with respect to extraction with fresh buffer. Effect of the overall mole fraction x (in the system) as shown. For x see legend of Fig. 5. Concentration of incubating solution: highest value of c_{VBLS} in the plots.

TABLE 4

KINETICS OF VBLS BINDING TO EL LIPOSOMES *, BY INCUBATION

t (days)	$(\frac{VBLS}{EL})_{enc}$	c_{VBLS} (M)	K_{app} $l \cdot mol^{-1}$)
1	0.028	2.1×10^{-4}	130
4	0.025	2.1×10^{-4}	120
5	0.027	1.8×10^{-4}	150
6	0.021	2.3×10^{-4}	90
7	0.026	2.0×10^{-4}	130

* The liposomes were obtained from vesicles frozen down to −19°C. c_{VBLS} (overall): 6.2×10^{-4} M. EL: 2.2×10^{-2} M. t: time of incubation. x = 0.03 (mol%) (in the system). c_{VBLS} = VBLS concentration inside supernatant.

stability towards supernatant dilution displayed also by the incubated (with VBLS) liposomes demonstrates the reversibility of the mechanism of VBLS encapsulation at large values of VBLS/EL ratios.

For the lowest x = 0.03 value, the drug encapsulation by incubation was studied also with liposomes obtained from vesicle frozen at −19°C for 24 h. The results of these incubation experiments carried out up to 7 days, are reported in Table 4. They were time-independent and consistent with those reported in Table 3b, allowing for the reproducibility of these experiments. When we consider the vesicle (Table 1) and liposome (Tables 3 and 4) encapsulated drug concentrations we notice that, in contrast to liposomes, the amount of encapsulated VBLS by vesicles was independent of its ab initio mole fraction x and that it saturates at low levels (~ 1‰–3‰, mol/mol). Such levels have been reported also for small sonicated liposomes (Juliano and Stamp, 1978; Layton et al., 1979). Therefore, under given conditions, large liposomes, whatever their method of preparation, when incubated with VBLS solution, encapsulated the drug faster than the vesicles and in larger amounts.

Discussion and Conclusion

VBLS encapsulation in vesicles

The outstanding result for VBLS vesicle encapsulation is the encapsulated drug low saturation level corresponding to 1‰–3‰ mole fraction in vesicles and about 10^{-5} M in the aqueous phase. The free drug is cytotoxic at lower concentrations (Owellen et al., 1972), the interactions with tubulin being significant at 10^{-7}–10^{-6} M. Similar low levels of encapsulation associated with a 10% efficiency of VBLS encapsulation have been reported earlier for small liposomes, but the external, free VBLS concentrations were not known. As our measured values of $(VBLS/EL)_{enc}$ are independent of vesicle composition, of the ab initio VBLS vesicle and aqueous phase composition we believe that the $K_{app} \simeq 100$ (see Table 1), corresponds to the equilibrium partition of the drug *molecules* between EL vesicles and dilute (about

10^{-5} M) aqueous phase. The ideal thermodynamic partition coefficient K_{id} defined as follows:

$$K_{id} = \frac{x_{enc}}{x_w} \tag{1}$$

where x_w and x_{enc} are the drug mole fractions in the vesicle and aqueous phases, respectively, has been recalculated. It is equal to $K_{id} \simeq 5550$. Allowing for the ratio of molar volumes of water and octanol, the values $K_{id} \simeq 16{,}500$ and $K_{id} \simeq 825$ are recalculated from Eqn. 1, respectively, for the *free base* VBL and for VBLS partition between octanol and a phosphate buffer (pH = 7.3). Finally, unloaded EL vesicles, brought into contact with a concentrated drug solution, $c_{VBLS} = 6.2 \times 10^{-4}$ M, first saturate rapidly with VBLS molecules ($x_{enc} \simeq 10^{-3}$). Additional depletion of the aqueous phase (Fig. 3a) may imply either drug influx (into the vesicle internal aqueous volume v_v) or/and association with vesicle wall. The v_v being about 0.4–0.5% of the dispersion total volume and the depletion of VBLS solution being as high as 14% in some cases (Fig. 3a), we rejected the hypothesis of VBLS influx. In contrast to the conclusion of Juliano and Stamp (1979), who studied VBLS efflux from loaded dimyristoylphosphatidylcholine, we concluded that additional drug association with the vesicle wall took place and increased the value of K_{app}. It has been reported (Nimni, 1972) that at pH = 7.4 and t > 25°C, strong aggregation of VBLS occurs in concentrated ($\simeq 10^{-3}$ M) aqueous solutions and that association of VBLS with amphiphiles (detergent, protein) substitutes to this aggregation. It is possible that at 4°C, the slow rate vesicle–drug association observed, in our 6.2×10^{-4} M VBLS solution, has substituted to the drug aggregation.

VBLS encapsulation in liposomes

The outstanding feature of this series of results is the large drug encapsulating efficiency and levels obtained by raising the ab initio VBLS level in the system. A similar behaviour has been reported recently (Crommelin and Van Bloois, 1983) for another drug, doxorubicin. For VBLS the high ab initio level also improved the encapsulation stability with respect to dilution of the loaded liposome dispersion under given conditions (see Table 3a and b). Furthermore, the high loading capacity appears independent of the ab initio drug location (inside either the aqueous or the lipid phases) provided the overall drug concentration is high. Thus the apparent and thermodynamic partition coefficients of VBLS between EL and an aqueous phase at pH = 7.3 are in the range $200 < K_{app} < 400$ [$l \cdot mol^{-1}$] or $11{,}000 < K_{id} < 22{,}000$, respectively, and levels as high as $x_{enc} = 10\%$ molar of drug encapsulation have been attained for liposomes. The reason for the different EL–VBLS miscibility in vesicles and in liposomes is not clear. We may speculate on the basis of results obtained by one of us (Ter-Minassian-Saraga and Madelmont, 1981, 1983) for the fully hydrated mixed DPPC–VBLS bilayers. It was found that at low mole fractions x = 0.1, VBLS decreased the ordered DPPC domain size of the bilayers and that at x > 0.2 a phase separation occurred in the fluid ones. The presently studied EL multi-bilayers are fluid. The results in Fig. 5a and b imply that a cooperative-like encapsulation of VBLS occurs because the concentration of encapsulated VBLS increases with x. It

has been shown (Op den Kamp et al., 1974) that liposome heterogeneity, such as a thermal transition, enhances protein binding. It is possible that, for our lecithin liposomes, an isothermal phase separation, induced by the solubilized VBLS favours the entrappment of segregated drug between bilayer domains and/or between bilayers. Our results of "rinsing" experiments (Table 3a and b) indicate that this additionally pseudo-encapsulated drug is released slowly. Unfortunately the equilibrium concentration of free VBLS in the first supernatant ($\simeq 10^{-4}$ M) is well above this drug toxicity level. Nevertheless if the drug clearance were rapid, the free VBLS stationary concentration would be inferior to the drug toxicity level. Under these circumstances the loaded liposomes might be superior, for VBLS delivery, to the vesicles.

Acknowledgements

We thank Prof. E. Taillandier and Dr. A. Cao for measuring the vesicle hydrodynamic radius and E. Rouère for the HPC chromatography of the phospholipids.

References

Crommelin, D.J.A. and van Bloois, L., Preparation and characterization of doxorubicin-containing liposomes. II. Loading capacity, long-term stability and doxorubicin–bilayer interaction mechanism. Int. J. Pharm., 17 (1983) 135–144.

Eibl, H. and Lands, W.E., A new, sensitive determination of phosphate. Anal. Biochem., 30 (1969) 51–57.

Juliano, R.L. and Stamp, D., Pharmacokinetics of liposome-encapsulated anti-tumor drug. Biochem. Pharmacol., 27 (1978) 21–27.

Juliano, R.L. and Stamp, D., Interactions of drugs with lipid membranes. Characteristics of liposomes containing polar or non-polar anti-tumor drugs. Biochim. Biophys. Acta, 586 (1979) 137–145.

Layton, D., De Meyere, J., Collard, M.P. and Trouet, A., The accumulation by fibroblasts of liposomally encapsulated vinblastine. Eur. J. Cancer, 15 (1979) 1475–1483.

Layton, D. and Trouet, A., A comparison of the therapeutic effects of free and liposomally encapsulated vincristine in leukemic mice. Eur. J. Cancer, 16 (1980) 945–950.

Milsmann, H.W., Schwendener, R.A. and Weder, H.G., The preparation of large single bilayer liposomes by a fast and controlled dialysis. Biochim. Biophys. Acta, 512 (1978) 147–155.

Nimni, M.E., Vinblastine sulphate, its reversible thermal aggregation and interaction with hydrophobic groups. Biochem. Pharmacol., 21 (1972) 485–493.

Nozaki, Y., Lasic, D.D., Tanford, C.H. and Reynolds, J.A., Size analysis of phospholipid vesicle preparations. Science, 217 (1982) 366–367.

Op den Kamp, J.A.F., De Gier, J. and van Deenen, L.L.M., Hydrolysis of phosphatidylcholine liposomes by pancreatic phospholipase A_2 at the transition temperature. Biochim. Biophys. Acta, 345 (1974) 253–256.

Owellen, R.J., Owens, A.H. and Donigian, D.W., The binding of vincristine, vinblastine and colchicine to tubulin. Biochem. Biophys. Res. Commun., 47 (1972) 685–691.

Owellen, R.J., Donigian, D.W., Hartke, C.A. and Hains, F.O., Correlation of biologic data with physico-chemical properties among the vinca alkaloids and their congeners. Biochem. Pharmacol., 26 (1977) 1213–1219.

Parsegian, V.A., Fuller, N. and Rand, R.P., Measured work of deformation and repulsion of lecithin bilayers. Proc. Natl. Acad. Sci. U.S.A., 76 (1979) 2750–2754.

Ter-Minassian-Saraga, L., Madelmont, G., Hort-Legrand, C. and Métral, S., Vinblastine and vincristine action on gel–fluid transition of hydrated DPPC. Biochem. Pharmacol., 30 (1981) 411–415.

Ter-Minassian-Saraga, L. and Madelmont, G., Enhanced hydration of dipalmitoylphosphatidylcholine multibilayer by vinblastine sulphate. Biochim. Biophys. Acta, 728 (1983) 394–402.