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Preparation and characterization of doxorubicin-containing liposomes: I. Influence of liposome charge and pH of hydration medium on loading capacity and particle size

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Summary

Physicochemical characterization of doxorubicin-containing liposomes is essential to obtain reproducible results in in vivo studies. Particle size, loading capacity and release on storage of doxorubicin liposomes were investigated in different stages of liposome preparation as a function of buffer pH (4, 6.3, 7.4, 8.4) and liposome composition: negatively or positively charged. It was found that doxorubicin strongly interacted with both types of liposomes. For the higher pH dispersions non-liposomal structures of unknown composition were suspected. Filtration as sizing procedure was only effective for negative liposomes at low pH. Both positively and negatively charged vesicles could be effectively reduced in size by ultrasonication in the pH range from 4 to 6.3. Release rates of the drug were low under these conditions. For preparation of well-characterized liposome dispersions with doxorubicin this pH range is to be preferred.

Introduction

Liposomes may become extremely useful as drug carriers or targeting agents as they can extensively change the distribution of the enclosed drug compound over the body. Problems to be solved concentrate firstly around reducing the affinity of the reticuloendothelial system for liposomes to improve targeting efficiency and secondly around increasing the loading capacity. Because particle size, charge and

chemical composition are factors influencing the behaviour of the liposomes, and thereby the associated drug compound, attention has to be paid to manufacture well-characterized and stable liposomes (Kimelberg et al.,1975; Juliano et al., 1978; Rustum et al., 1979; Ryman and Tyrrell, 1979; Szoka and Papahadjopoulos, 1981; Fildes, 1981).

The therapeutic use of doxorubicin, and antitumor agent, is limited by its concurrent cardiotoxicity. Recently 4 different research groups (Rahman et al., 1980; Forssen and Tökes, 1980; Gabizon et al., 1982; Olson et al., 1982) have reported their results obtained with doxorubicin loaded in liposomes studying both antitumor effect and cardiotoxicity in rodents. Although the results of the 4 above-mentioned studies show substantial differences, the overall conclusion is that liposomes can increase the therapeutic index of doxorubicin.

Only in the last-mentioned publication was attention paid to particle size and stability. But no systematic study was done to find optimal conditions for the preparation of doxorubicin-containing liposomes. Our objective was to prepare liposomes with narrow particle size distributions, stable against agglomeration or fusion on storage combined with a high loading capacity, limited drug release on storage and a known charge. In this article we report on the particle size, loading capacity and release on storage of doxorubicin-containing liposomes prepared under different conditions. The liposome compositions corresponded to those used in the study of Rahman et al. (1980). Charge, pH of the hydration buffer and phospholipid (PL) concentration were variables under investigation.

Methods and Materials

Preparation of liposomes

Positive liposomes consisted of egg L-α-phosphatidylcholine (PC) (type V-E, Sigma Chemicals, St. Louis, MO), cholesterol (Sigma Chemicals, St. Louis, MO) and stearylamine (SA) (ICN Pharmaceuticals, Plainview, NY) in the mol. ratio 50 : 20 : 15. For negative liposomes a mol. ratio PC : cholesterol : L-α-phosphatidylserine (PS) (bovine brain, Sigma Chemicals, St. Louis, MO) of 50 : 20 : 5 was used. These ratios were taken from Rahman et al. (1980). The general procedure of preparation was as follows: Adriablastine (Farmitalia), containing doxorubicin with lactose (1 : 5), was mixed with PC, cholesterol, and SA or PS in about 5 ml of a chloroform/methanol 1 : 1 mixture (reagent grade) in a 50 ml pear-shaped flask and rotary evaporated under low pressure at 40–50°C to yield a film. The flask was left under vacuum for at least 2 h. The medium for hydrating the film consisted of 0.01 M tris buffer with 0.8% sodium chloride, and with 0.05 mM EDTA in the case of negative liposomes. The water used was freshly prepared by distillation from glass. Nitrogen was passed through the buffer for 15 min before film hydration. When necessary the pH was adjusted to the initial value by adding dilute hydrochloric acid or sodium hydroxide solutions. After addition of glass beads the films were hydrated by handshaking and vortexing at 45°C and left after complete dispersion of the film for at least 2 h at

room temperature. As a general rule all dispersions and solutions containing doxorubicin were stored protected from light at 4–6°C.

Sizing of liposomes by filtration

At least one day after dispersing, the liposomes — mainly MLV (multilamellar vesicles) — were sequentially filtered through 0.6 and 0.2 μm Nucleopore membrane filters (Olson et al., 1979; Szoka et al., 1980). Pressures up to 600 kPa had to be used. If necessary the pH was readjusted.

Preparation of small unilamellar vesicles (SUV)

Nitrogen was passed through the filtered liposome dispersions for 5 min under cooling in ice-water. Thereafter the dispersions were sonicated (Bransonic B12, probe type sonicator) in consecutive 2 min bursts separated by a 1 min rest interval. Sonication continued until the turbidity did not decrease anymore. Total actual sonication time was 10–12 min at most. Nitrogen was passed over the dispersion continuously and it was cooled in ice-water to prevent chemical degradation of the phospholipids during sonication (Hauser, 1971). In some cases these dispersions were ultracentrifugated at 10^5 *g* for 1 h to remove multilamellar structures completely.

Separation of free from liposome-associated doxorubicin

Two methods were used to separate free from liposome-associated doxorubicin.

Dialysis: analytical and preparative purposes. The dispersions were dialyzed at 4°C against 100–125 ml of buffer of corresponding composition. Sink conditions were maintained throughout the dialysis process. Both donor (maximum 5 ml) and acceptor compartment were stirred. The dispersions were protected from light. The dialysis membranes (M_r 10,000 cut off, 3.2 cm^2 exchange area, Diachema, Rüschlikon, Zürich) were soaked with distilled water for at least 15 min and extensively rinsed before use. In this set up a plain doxorubicin solution (1 $mg \cdot ml^{-1}$) in buffer in the donor compartment practically reached equilibrium with the receptor compartment within 5 h.

Ultracentrifugation: analytical purposes. This method could only be used for dispersions containing MLV (filtered or non-filtered). The dispersions were centrifuged at 10^5 *g* at 4°C for 1 h. Total doxorubicin and phospholipid concentrations were determined before and after centrifugation (in the supernatant) and from these data the ratio associated doxorubicin to total doxorubicin or the amount of doxorubicin associated related to the amount of phospholipid present was calculated. The assumption was made that the precipitate only consisted of liposomal material. As will be shown later this assumption held only in a limited pH range.

Analytical methods

Doxorubicin was assayed spectrophotometrically at 480 nm. Up to absorption readings of 1.0 Beer-Lambert's law was applicable. Total doxorubicin (free plus associated) was determined after adding Triton X-100 ($< 1\%$) to the dispersion and vortexing until the turbidity measured at 700 nm was negligible. In some cases it was

necessary to centrifuge the Triton X-100-containing solutions for 15–30 min at 700 *g* to precipitate interfering solid material. Addition of Triton X-100 to doxorubicin solutions did not influence the absorption readings. Phospholipid concentrations were determined by measuring phosphate concentrations using the method described by Fiske and SubbaRow (1925).

Turbidity was measured spectrophotometrically at 700 nm. Doxorubicin absorption is negligible at this wavelength.

Release of associated doxorubicin from liposomes

To monitor the release rate of doxorubicin from filtered MLV, 0.5 ml samples of a dispersion of liposomes with doxorubicin were diluted with 10 ml of the corresponding buffer. They were stored at 4–6°C without shaking. At predetermined time intervals one diluted sample was taken and the ratio free to liposome-associated doxorubicin determined by ultracentrifugation.

Size analysis

For electron microscopic observations (Philips EM 201) of the samples the negative staining technique was used. Staining of the samples was done in a two-step procedure. After dilution of the samples with 0.1 M ammonium acetate solution a drop was mixed with a 1% ammonium molybdate solution on a Cu-grid 300 mesh and dried. For dynamic light-scattering measurements a Nanosizer (Coulter Electronics) was used. With this instrument the mean diameter and a polydispersity index can be obtained. This index ranges from 0 to 9. Zero indicates to a monodisperse and 9 to an extremely polydisperse system. As an example for orientation: a gold sol approaching a normal size distribution with a mean diameter of about 50 nm and a coefficient of variation of about 15%, has a polydispersity index of 3 (Leuvering, personal communication).

Results and Discussion

Successively the following subjects will be treated: (1) loading efficiency; (2) release of doxorubicin from the liposomes; and (3) particle size in different stages of liposome preparation.

Loading efficiency: filtered liposomes without pH adjustment

Dispersing the phospholipid film (final PL concentration 20 $\mu mol \cdot ml^{-1}$) with doxorubicin in the Tris · HCl–sodium chloride solution of pH 7.4 resulted in a slight increase to pH 7.6 in case of positive liposomes. For negative liposomes, however, the pH dropped sharply to values below pH 5. During dialysis the pH of both liposome dispersions approached the pH (7.4) of the dialysis medium. The reproducibility of the loading capacity of doxorubicin was studied by preparing a number of dispersions consecutively at 2 or 3 day intervals using chemicals from the same batches. The doxorubicin associated per mol PL, in case no pH adjustments were made before dialysis, is given in Table 1.

TABLE 1

DOXORUBICIN ASSOCIATED (mmol) PER MOL PHOSPHOLIPID AS CALCULATED FROM ULTRACENTRIFUGE DATA; NO pH ADJUSTMENTS

Charge	Experiment				Mean	Coefficient of variation (%)
	I	II	III	IV		
+	37	42	48	n.a.	42	13
−	57	35	40	40	43	22

Initial doxorubicin concentration 2 μmol·ml^{-1}; phospholipid concentration about 20 μmol·ml^{-1}; volume of suspension 15 ml; liposomes were filtered through 600 and 200 nm membrane filters and dialyzed for at least 30 h at 4–6°C; pH of dialyzing medium: 7.4.
n.a. = not available.

As can be seen, even under standardized conditions a substantial spreading in binding capacity data was found.

Loading efficiency: filtered liposomes with pH control

It was investigated to what extent the loading efficiency depended on the pH of the buffer. Positive or negative liposomes were prepared to assess the loading capacity of liposomes for doxorubicin in the pH range between 4 and 8.4. The pH was controlled within narrow limits ($\pm$0.1) by re-adjusting when necessary. A PL concentration of about 6 μmol · ml^{-1} was used. Fig. 1 shows the resulting doxorubicin association, expressed as mmol doxorubicin bound per mol PL for non-filtered and filtered liposomes, for positive liposomes also after dialysis.

For filtration of 6 μmol PL · ml^{-1} dispersions through 0.2 μm pores pressures of 600 kPa were needed. In particular positive liposomes were difficult to pass through. For negative liposomes it was shown that for pH 7.4 and 8.4 the loading capacity as measured by the ultracentrifugation technique increased during the first day after dispersion. For pH 4 and 6.3 no increase was found. After filtration the capacity decreased for the 3 lower pHs (4, 6.3, 7.4), but increased for pH 8.4. For negative liposomes the binding capacity was only determined after dialysis for the pH 8.4 liposomes. A 7% loss was found after 6 days of dialysis. The ratio of total doxorubicin to phospholipid (mol. basis) in the dispersions differed between the experiments without pH control (Table 1) and with pH control (Fig. 1) being about 0.1 and 0.3, respectively. For the negative liposomes the loading capacity increased with increasing ratio. For 3 consecutive, non-filtered, batches with a constant doxorubicin-to-phospholipid ratio of 0.1 and under pH control (pH 4), a mean loading capacity of 35 mmol doxorubicin/mol PL with a coefficient of variation of 5% was found.

Data of doxorubicin association with positive liposomes suggested an analogous pH-dependent increase of loading capacity as a function of time. Data collected for non-filtered, non-dialyzed dispersions at pH 4 and 6.3 did not change during the first 3 days after preparation. If the doxorubicin to phospholipid ratio exceeded a value of about 0.1 a constant loading capacity was found: 50 mmol doxorubicin/mol

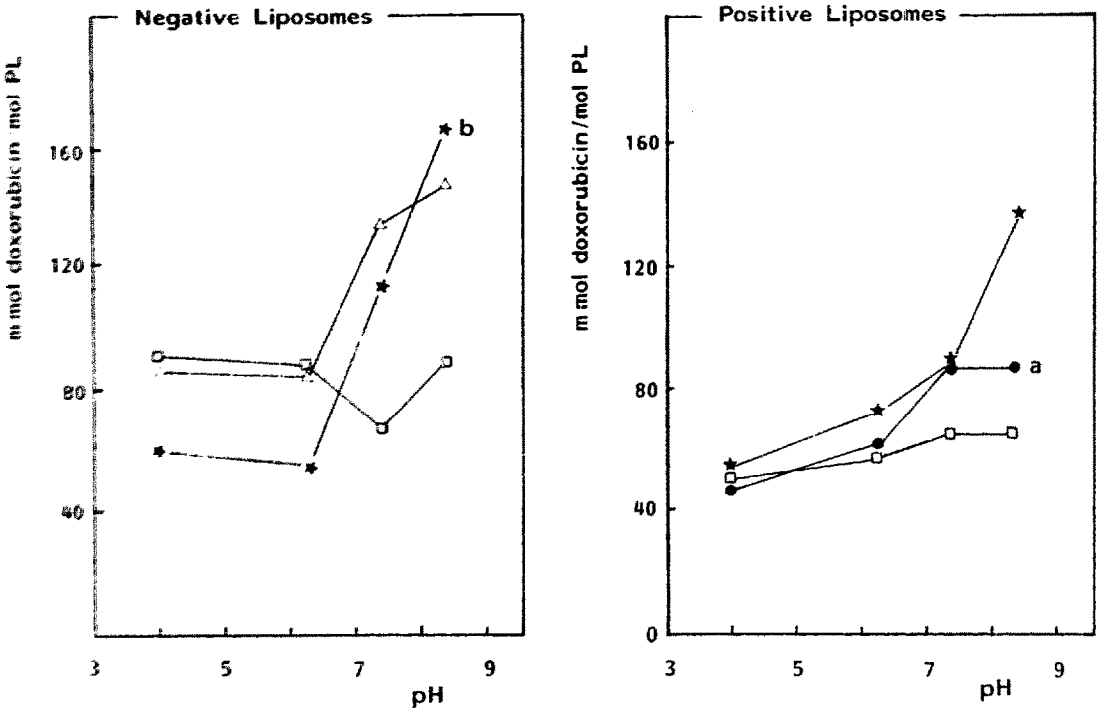

Fig. 1. Mmol doxorubicin associated per mol phospholipid calculated from the ultracentrifuge technique data. Initial concentration doxorubicin: 2 μmol · ml^{-1}; concentration phospholipid 6 μmol · ml^{-1}; volume of suspension 10 ml; n = 2. Negative liposomes. □, day of preparation; △, 24 h after hydration; ★, 24 h after hydration, filtered; (b) after dialysis for 2 days = 150 mmol per mol PL; and for 6 days = 150 mmol per mol PL. Positive liposomes. (a) Precipitate on dialysis membrane. □, non-filtered; ★, filtered. Filtration at least 24 h after hydration. ●, dialyzed for 140 h, filtered, dialyzed.

phospholipid with a coefficient of variation of 13% (n = 12). This data was obtained for phospholipid concentrations in the range between 3 and 30 μmol · ml^{-1}. Generally spoken, dialysis did not reduce the loading capacity for positive liposomes. An exceptional behaviour was observed with positive liposomes at pH 8.4. Here a precipitate was found on the dialysis membrane after dialysis and the amount of doxorubicin associated per mol PL, as measured by the ultracentrifugation technique, dropped. It was found that even after 140 h of dialysis, after 4 replacements of the acceptor medium buffer and no remaining net transport across the membrane, still free doxorubicin (up to 30%) could be present in the donor compartment. Olson et al. (1982) also found one membrane insufficient for complete separation of free from liposome-associated doxorubicin. They recommend a repeated renewal of the membrane. It is our experience, too, that renewal of membranes resulted in a more complete removal of free drug from the dispersions. In our set up doxorubicin crossed the membrane easily in the absence of phospholipids. The reason for the poor exchange of doxorubicin in the presence of liposomes was not further investigated yet. This finding stresses the point of checking the extent of free drug removal after dialysis even if - under sink conditions - transport through the membrane seems to be finished.

The recovery after filtration, expressed as percentage of the starting amount of doxorubicin or phospholipid before membrane passage, decreased from 80–90% for positive liposomes at pH 4 and 6.3, to 70% at pH 7.4 down to 50% at pH 8.4 for *doxorubicin*. For negative liposomes these numbers were 100–85%, 95% and 55%, respectively. Filtration did not decrease the *phospholipid* concentration at pH 4, 6.3 and 7.4 by more than 20%. For pH 8.4 about 90% and 75% of the phosphate was recovered after filtration for positive and negative liposomes, respectively. Thus at this high pH the loss of doxorubicin cannot be accounted for by retention of liposome-associated drug material on the filter alone.

Additional evidence of the presence of doxorubicin not associated with liposomal phospholipid material but in some other structure at pH 8.4 was collected by evaluating the concentrations of doxorubicin and phosphate left in the supernatant after sonication and ultracentrifugation. Table 2 gives the percentages of doxorubicin and phosphate remaining after ultrasonication and ultracentrifugation at pH 4 and 8.4 for both positive and negative, filtered, liposomes.

At pH 4 for positive liposomes, slightly more doxorubicin was precipitated than expected assuming only doxorubicin associated with liposomes being removed. These calculations were based on the loading capacity of the filtered liposomes. As this capacity is usually smaller for SUV than for MLV, too high values of doxorubicin left in the supernatant were expected. For negative liposomes the results agreed well with the results obtained for doxorubicin binding to phospholipid before ultrasonication and ultracentrifugation at pH 4. This situation was different for pH 8.4. Here much more doxorubicin precipitated than was expected on the basis of calculations assuming only liposome-associated doxorubicin to sediment. This means that the precipitate at pH 8.4 contained doxorubicin not associated with phospholipids or in a way different from the situation at pH 4.

The exact nature of the time-dependent increase in apparent loading capacity is not known. The formation of degradation products of doxorubicin or precipitation of doxorubicin itself might account for the phenomenon. Sturgeon and Schulman (1977) investigated doxorubicin in solution as a function of pH and concentration. Below pH 8 doxorubicin is mainly in protonated, positively charged, state in solution. Above pH 10 the negatively charged form dominates. The solubility is therefore highly pH-dependent. Other complicating factors are the occurrence of

TABLE 2

PERCENTAGE OF PHOSPHOLIPID (P-LEFT) AND DOXORUBICIN (DOXO-LEFT) IN THE SUPERNATANT AFTER ULTRACENTRIFUGATION OF SONICATED LIPOSOME DISPERSIONS AT pH 4 AND pH 8.4

pH	Negative liposomes (n = 2)		Positive liposomes (n = 2)	
	P-left (%)	Doxo-left (%)	P-left (%)	Doxo-left (%)
4	72	94	91	84
8.4	35	15	69	21

Concentration PL in dispersions: 1.5–2.5 $\mu mol \cdot ml^{-1}$; doxorubicin 0.3–0.6 $\mu mol \cdot ml^{-1}$.

dimers at concentrations as used in this study and the formation of degradation products. We determined whether the concentrations of doxorubicin at pH 8.4 exceeded the doxorubicin solubility as this might explain the pH-dependent increase in loading capacity with time. To this end 2 mg of doxorubicin was mixed with 1 ml of buffer. At room temperature a clear solution was readily obtained but soon precipitation started with a concurrent decrease in absorbance readings of the supernatant at 480 nm (n = 3). After 1 h the solutions were stored at 4–6°C and after 24 h the absorbance of the supernatant corresponded to less than 0.2 mg/ml doxorubicin. In analogous experiments with pH 4 solutions no precipitate was found. The increase in apparent loading capacity at pH 8.4 might therefore be caused by decomposition of the doxorubicin and precipitation of the formed product(s).

In conclusion the loading capacity data suggest that the pH range between pH 4 and 6.3 is to be preferred for liposome preparation as a time-independent loading was found, together with a high doxorubicin recovery and a low phospholipid retention on the membrane filter.

Influence of film thickness on loading capacity

Film formation on the glass wall might be a critical point in the preparation procedure. Therefore the influence of the film thickness on the loading capacity was investigated (Table 3) for positive liposomes by using one type of pear-shaped flasks (50 ml), and changing the phospholipid concentration, while keeping the ratio between the concentrations of doxorubicin and phospholipid constant. Apparently in the concentration range studied the loading capacity did not depend on film thickness.

Release of doxorubicin from the liposomes

For two different pH values the release of doxorubicin was determined at 4°C. The results are shown in Fig. 2. Both for positive and negative liposomes doxorubicin appeared to be firmly associated with the bilayer.

TABLE 3

INFLUENCE OF FILM THICKNESS ON THE LOADING CAPACITY OF POSITIVE LIPOSOMES FOR DOXORUBICIN (DOXO)

PL concentration (μmol·ml^{-1} buffer)	Mmol doxo loaded per mol PL	Number of determinations
3	46	n = 2 [a]
6	53	n = 2 [a]
20	46	n = 2 [b]

Constant ratio doxorubicin to phospholipid: about 0.2 (mol basis). Loading capacity did not change during a 3–4-day period of storage of the dispersions at 4–6°C. pH of the buffer is 4; non-filtered; non-sonicated

[a] Mean of day 1, 2 and 3 for each dispersion.

[b] Mean of day 1 and 4.

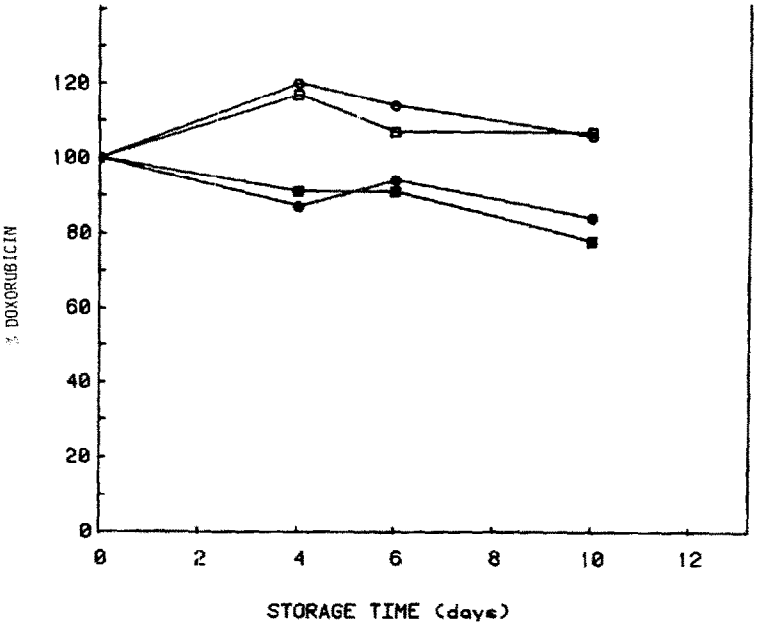

Fig. 2. Release of doxorubicin from positively or negatively charged liposomes over a 10-day time span at 4°C. Positive liposomes filtered through 200 nm membrane filters; non-dialyzed; storage temperature 4°C; total doxorubicin 5.5×10^{-2} μmol·ml^{-1}; PL: 0.2 μmol·ml^{-1}, n = 2. Negative liposomes filtered through 200 nm membrane filters; non-dialyzed; storage temperature 4°C; total doxorubicin: 9.2×10^{-2} μmol·ml^{-1}; PL: 0.3 μmol·ml^{-1}, n = 2. □, positive liposomes, pH 4; ○, positive liposomes, pH 6.3; ■, negative liposomes, pH 4; ●, negative liposomes, pH 6.3. % bound relates to the situation at t = 0.

Particle size in different stages of preparation

An impression of changes in particle size is obtained by monitoring the specific turbidity (A_{spec}), defined as the absorption per cm path-length at 700 nm per μmol PL · ml^{-1}, at different stages of the preparation process. For particles of similar composition with sizes below about 3 μm a decrease in turbidity generally indicates an overall size reduction (Kerker, 1969). In Table 4 some reference points are shown roughly indicating the relation between A_{spec} and mean particle diameters obtained by a dynamic light scattering technique. Non-filtered and non-sonicated dispersions were so heterodisperse that no meaningful dynamic light-scattering data could be obtained. The specific absorption as a function of the buffer pH in different stages of the liposome manipulation process is presented in Fig. 3.

For positive liposomes over the whole pH range a small and variable reduction of A_{spec} was found after filtration. For non-filtered liposomes A_{spec} showed a tendency to increase with pH. A substantial reduction of A_{spec} was observed for the negative liposomes at pH 4; for pH 6.3 filtration decreased A_{spec} less. Here A_{spec} was lower for non-filtered dispersions of pH 7.4, 7.9 and 8.4 than for pH 4 and 6.3, but A_{spec} increased on filtration of these high pH dispersions. Observations by light microscope revealed that, after filtration through 600 and 200 nm membrane filters, the dispersions, with the exception of negative liposomes at pH 4 and 6.3, contained a number of particles with diameters up to about 10 μm. Electron microscopic

TABLE 4

RELATION BETWEEN A_{spec} AND MEAN DIAMETERS OF LIPOSOME DISPERSIONS

Dispersion type [a]	Charge	A_{spec} ($ml \cdot \mu mol^{-1}$ PL $\cdot cm^{-1}$)	mean diameter [b] (μm)
Filtered (600, 200 nm) dialyzed	−	0.30	0.26
Sonicated, ultracentrifuged, dialyzed	−	0.030	0.12
Sonicated, ultracentrifuged	+	0.010	0.08

[a] pH 4.
[b] Coulter Nanosizer.
Polydispersity index of these dispersions: 2–3.

observations indicated that for the pH 7.4 and 8.4 dispersions the majority of the particles had dimensions between 500 and 1000 nm. So, light microscopic, electron microscopic and turbidity measurements agreed in so far that filtration did not reduce the particle size permanently to dimensions as small as the pore size of the membrane filter (200 nm).

To check our filtration technique we filtered "empty" positive and negative liposomes containing no doxorubicin but for the rest of similar composition and PL

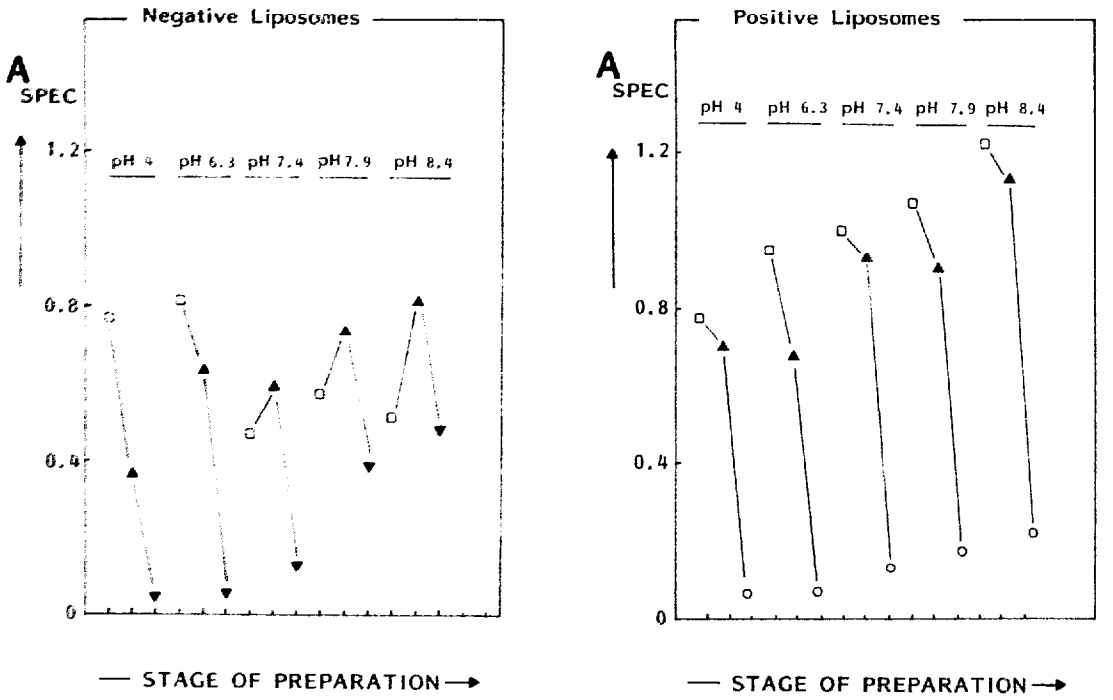

Fig. 3. Specific absorption (A_{spec}) in different stages of the liposome preparation process as a function of pH. Wavelength 700 nm, n = 2. □, non-filtered; PL concentration 5–9 $\mu mol \cdot ml^{-1}$; ▲, filtered through 200 nm; PL concentration 5–9 $\mu mol \cdot ml^{-1}$; ○, ▼, ultrasonicated for at least 8 min, PL concentration about 2 $\mu mol \cdot ml^{-1}$.

concentration through 600 and 200 nm filters and determined the resulting A_{spec} and mean diameter by dynamic light-scattering. The results are shown in Table 5.

For positive and negative liposomes, filtration brought about a substantial size reduction at both pH values as shown by both measuring techniques. The dynamic light-scattering data also indicated a strong decrease in polydispersity.

For both positive and negative doxorubicin-containing liposomes A_{spec} measured after ultrasonication increased with pH (Fig. 3). This effect was especially clear for the negative liposomes. Fig. 4 shows the A_{spec} of the liposome dispersions as a function of the sonication time. For pH 4 a low, constant level was reached within 4 min. For pH 7.9 and 8.4 dispersions A_{spec} became constant after 2 min at a relatively high level (pH 7.9) or still slowly decreased to this high level (pH 8.4) after 8 min of sonication.

The remaining turbidity did not disappear on Triton-X addition supporting the suggestion made above, that at pH values larger than 7.4 non-liposomal structures are formed. The sonicated dispersions of pH 4 were ultracentrifuged for 1 h at 10^5 *g* to precipitate multilamellar structures. The reduction of the phospholipid concentration in the supernatant was less than 10%. Within one day after ultracentrifugation no multilamellar structures could be observed by the electron microscope in the supernatant; for both positive and negative liposomes mean particle diameters

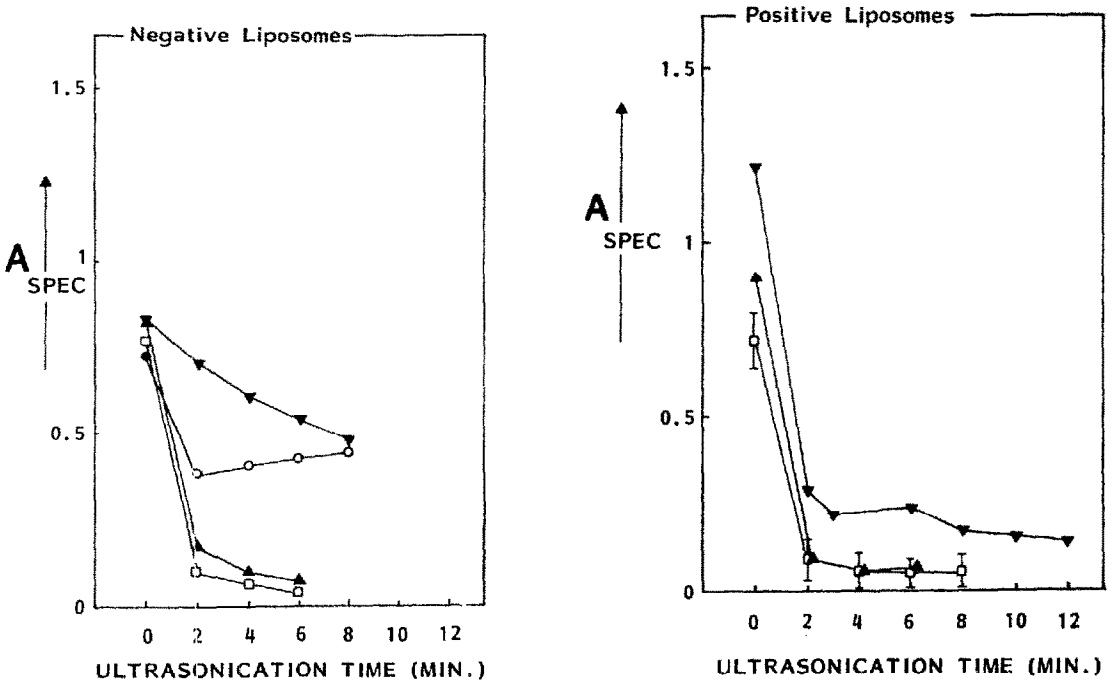

Fig. 4. Effect of ultrasonication on specific absorption (A_{spec}) as a function of pH. Negative liposomes: □, pH 4 filtered (n = 2); ▲, pH 6.3 filtered (n = 2); ○, pH 7.9 filtered (n = 2); ▼ pH 8.4 filtered (n = 2). Phospholipid concentration about 2 $\mu mol \cdot ml^{-1}$. Positive liposomes: □, pH 4 mean of filtered dispersions (n = 4) (vertical bars represent standard deviation); ▲, pH 6.3 filtered (n = 2); ▼, pH 8.4 filtered (n = 2). Phospholipid concentration about 2 $\mu mol \cdot ml^{-1}$.

TABLE 5

EFFECT OF FILTRATION (600, 200 nm) ON PARTICLE SIZE FOR "EMPTY" LIPOSOME DISPERSIONS (NO DOXORUBICIN) FOR pH 4 AND pH 8.4

pH	Charge liposomes	A_{spec}		mean diameter (μm) [a]	
		(b)	(c)	(b)	(c)
4.0	+	1.89	0.43	*	0.26
8.4	+	2.05	0.31	*	0.24
4.0	−	2.01	0.58	*	0.27
8.4	−	2.40	0.42	*	0.26

Concentration PL about 5 μmol·ml^{-1}; n = 2.

[a] Nanosizer, Coulter Electronics.

[b] Before filtration through 600 and 200 nm Nucleopore membrane.

[c] After filtration through 600 and 200 nm Nucleopore membrane, polydispersity index: 2–3.

* Too heterodisperse for meaningful evaluation; polydispersity index ≥ 7. Average particle size > 1 μm.

around 100 nm were found. Results of dynamic light-scattering measurements agreed well with these findings.

Conclusions

Choosing the correct experimental conditions appears to be extremely important for the quality of the doxorubicin-containing liposome dispersions with respect to loading capacity, particle size and fraction of non-associated drug, and therefore for the therapeutic index of this drug in vivo. Doxorubicin was strongly bound to both positive and negative liposome membranes. It intercalated into or adhered to the bilayers up to 4–10 mol% of the total mass of PL involved and stayed in the liposomes during the observed period of storage. The exact mechanism of the pH-dependent loading capacity, as measured by the ultracentrifugation method, is not known yet; there is an indication of the presence of non-liposomal doxorubicin structures above buffer pH 7.4. An important finding was the pH- and charge-dependent efficacy of filtration of the liposomes. For positive liposomes filtration through 200 nm pores did not result in a substantial size reduction. Only for negative liposomes at pH 4 and 6.3 a size reduction was found with little retention of doxorubicin or phospholipid on the membrane filter. It is proposed that for preparation of doxorubicin-containing liposomes a slightly acidic medium should be preferred as liposome association at pH 4 and 6.3 was time independent with low release rates on storage. Filtration of the positive, doxorubicin-containing liposomes to obtain narrow size distributions, is useless as apparently due to fusion or agglomeration immediately after filtration "supra-pore" size structures were present in the dispersion. Reducing the amount of doxorubicin in the bilayer by loading the liposomes with sub-maximal amounts of the drug might solve this problem. Alternatively, with liposome dispersions at pH 4 and 6.3 (positive or negative) one can

obtain small particles by ultrasonication. After ultracentrifugation the supernatant contains SUV with diameters around 100 nm.

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Abbreviations

A_{spec}	specific absorption at 700 nm; absorption per $\mu mol \cdot ml^{-1} \cdot cm^{-1}$
MLV	multilamellar vesicles
n	number of experiments
PC	egg phosphatidylcholine
PL	phospholipids
PS	phosphatidylserine
SA	stearylamine
SUV	small unilamellar vesicles

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