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Doxorubicin induces aggregation of small negatively charged liposomes

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Abstract

The anticancer drug doxorubicin (DOX) can induce aggregation of liposomes containing a transmembrane ammonium sulfate gradient. This process proved to be dependent on several parameters. (1) Initial vesicle size. Aggregation of liposomes composed of dipalmitoylphosphatidylcholine (DPPC)/dipalmitoylphosphatidyl glycerol (DPPG)/cholesterol (CHOL) (10:1:4), molar ratio with an average diameter of 0.07 μm, was more pronounced than for those of 0.1 and 0.2 μm. (2) Doxorubicin (DOX)/phospholipid (PL) ratio. A certain threshold DOX/PL ratio was required to induce aggregation. (3) Composition of the bilayer. The shorter the fatty acid chain of the PC component, the higher the tendency to aggregate (dimiristoylphosphatidylcholine (DMPC) > DPPC > distearoylphosphatidylcholine (DSPC)). Besides, the addition of cholesterol increased the critical DOX/PL ratio required to induce aggregation. Interestingly, no aggregation was observed for egg phosphatidylcholine (EPC)/egg phosphatidylglycerol (EPG)/CHOL (10:1:4) liposomes. The presence and nature of the negative charge inducing lipid in the bilayer also played a role. Liposomes containing DPPG were more susceptible to aggregation than those containing dipalmitoylphosphatidylserine (DPPS), whereas neutral liposomes (DPPC/CHOL (10:4)) did not aggregate. If aggregation occurred with cholesterol free liposomes, it always happened after liposome loading with DOX at elevated temperatures, during the cooling down process, when liposomes reached their phase transition temperature. Then, we suggest that non-entrapped doxorubicin binds to the lipid bilayer (mainly by electrostatic interactions), as well as to other doxorubicin molecules (stacking), which in turn interact with membranes of other liposomes, thus inducing aggregation.

Keywords: Acidic phospholipid; Bilayer composition; Doxorubicin; Liposomes; Phase transition temperature; Vesicle aggregation; Vesicle size

1. Introduction

The anthracycline antibiotic doxorubicin (DOX) is one of the most widely used anticancer agents. It has shown antineoplastic activity against acute leukemia, non-Hodgkin's disease, lung carcinomas and sarcomas. However, cardiac muscle damage is the major cause for dose limitation (for a recent review see Ref. [1]).

Liposomes, lipidic vesicles containing one or more internal aqueous spaces, are known to alter pharmacokinetics and biodistribution of the entrapped agents [2–4]. An early observation was that liposomes showed a distinct tendency to avoid accumulation in intact cardiac tissue. Therefore, a number of investigations have focussed on the potential of liposome-encapsulated DOX formulations to improve its therapeutic index, by reducing its cardiac toxicity while maintaining or improving the antitumor effect [5–7]. For a number of anticancer agents in liposomes the best antitumor activity has been found for liposomes exhibiting long circulation times [4,8–10]. In the particular case of DOX, it is known that antitumor drug potency increases with decreasing vesicle size [11]. Therefore, small liposomes are preferred for therapeutic purposes. In order to limit the lipid dose required, a high DOX/lipid ratio would be desirable.

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Efficient and stable loading of DOX into liposomes has been described by Haran et al. [12] by using transmembrane ammonium sulfate gradients. This approach is based on the DOX exchange with neutral ammonia, which rapidly diffuse from the intraliposomal to the external medium. Once inside the liposomes, the anthracycline sulfate salt is formed and accumulates in an aggregated and gelated state. These authors successfully prepared DOX-containing liposomes with a variety of lipid compositions and methods of preparation.

However, recent studies in our laboratory have shown that some small, negatively charged liposomes encapsulate less DOX and tend to aggregate rapidly upon DOX loading. As liposome size is vital in terms of therapeutic activity, in this paper the effect of a number of parameters on the aggregation of liposomes upon incubation with the anthracycline DOX has been investigated, and an attempt has been made to elucidate the mechanism behind this phenomenon. These parameters include: (1) vesicle size, (2) DOX/phospholipid ratio, (3) bilayer rigidity, (4) vesicle surface charge, (5) type of charge inducing lipid, (6) temperature, (7) loading efficiency and (8) extraliposomal DOX.

2. Materials and methods

2.1. Materials

DOX was a gift from Pharmachemie B.V. (Haarlem, Netherlands). Dipalmitoylphosphatidylcholine (DPPC), dimiristoyl-phosphatidylcholine (DMPC), distearoylphosphatidylcholine (DSPC) were obtained from Nattermann GmbH (Cologne, FRG), dipalmitoylphosphatidylglycerol (DPPG), egg phosphatidylcholine (EPC) and egg phosphatidylglycerol (EPG) were obtained from Lipoid (Ludwigshafen, FRG), cholesterol (CHOL) and distearoylphosphatidylglycerol (DSPG) were purchased from Sigma (St Louis, MO) and dipalmitoylphosphatidylserine (DPPS) was from Avanti Polar Lipids (Pelham, AL, USA). Other chemicals were commercial grade. Dowex 50W-X4 was obtained from Baker Chemicals (Philipsburg, NJ).

2.2. Liposome preparation

Liposomes of different compositions were prepared by the 'film method'. Briefly, the lipid film was hydrated above the transition temperature of the corresponding lipid mixture with a solution of 120 mM ammonium sulfate. The initial phospholipid concentration was 25 mM. The dispersion was extruded several times through polycarbonate filters of decreasing pore diameter to obtain liposomes with diameters of approximately 0.07, 0.1 and 0.2 μm. Loading of DOX was carried out by using the ammonium sulfate gradient as described by Haran et al. [12]. In order to obtain such a gradient, the extraliposomal aqueous medium was replaced by 10 mM Hepes pH 7.4 and 5% (w/v) glucose by means of gel filtration chromatography on a Biogel P-6 column. The most diluted liposomal fractions were discarded. The phospholipid (PL) concentration of the resulting dispersions, measured as described by Rouser [13], ranged from 20–25 mM. For a number of experiments, liposomes without an ammonium sulfate gradient were prepared. In this case, liposomes were directly hydrated with 10 mM Hepes buffer pH 7.4 and 5% glucose, pH 7.4. Subsequently, liposomes were extruded to a final size of approx 0.07 μm and characterized as described before.

2.3. Liposome loading with DOX

To a certain volume of each liposome dispersion, varying amounts of a DOX solution and 10 mM Hepes, 5% (w/v) glucose pH 7.4 were added so as to obtain μg DXR/μmol PL ratios ranging from 25 to 200. In all cases, the PL concentration in the incubation media was 10 mM. All samples were incubated for 30 min in a water bath at 40, 60, 65 and 70°C for EPC, DMPC, DPPC and DSPC containing liposomes, respectively. The loading process was ended by cooling down the samples at room temperature.

2.4. Liposome characterization

2.4.1. Mean vesicle size

Immediately after incubation with DOX, all preparations were checked for aggregation by visual inspection and by dynamic light scattering with a Malvern 4700 system using a 25 mW He–Ne laser and the Automeasure version 3.2 software (Malvern, Malvern, UK). As a measure of the particle size distribution of the dispersion, the system reports a polydispersity index. This index ranges from 0.0 for an entirely monodisperse up to 1.0 for a polydisperse dispersion.

2.4.2. DOX loading efficiency

A 50 μl sample of each dispersion was passed through a column filled with Dowex 50W-X4 in order to remove free DOX (liposomal fractions). In this step samples were diluted ten times. The same dilution was applied to 50 μl of each dispersion in which free DOX was not removed (total fractions) [14]. Percentage of entrapment was determined by comparison of the fluorescence (λ_{ex}: 493 nm; λ_{em}: 585 nm) between liposomal and total fractions, after disruption of liposomes with 2-propanol. Absolute concentrations were also calculated with a calibration curve.

2.5. Differential scanning calorimetry

Differential scanning calorimetry of liposomes composed of DMPC/DPPG, DMPC/DPPG/CHOL, DPPC/DPPG, DPPC/DPPG/CHOL, DSPC/DPPG, DSPC/DPPG/CHOL either loaded with DOX or without DOX was performed on a T.A. Instruments 2920 DSC. 25 μl aliquots of the liposome dispersions were put into aluminium pans. As a reference an empty aluminium pan was used. Calorimetric scans were performed from 0 to 70°C, at a scanning rate of 2°C/min. The system was calibrated with indium for enthalpy and with indium and gallium for temperature scale.

2.6. Determination of the temperature of aggregation

DMPC/DPPG/CHOL, DMPC/DPPG and DPPC/DPPG liposomes were incubated with DOX at a DOX/PL ratio of 100 μg/μmol under the same conditions described before. DPPC/DPPG/CHOL and DSPC/DPPG liposomes were also incubated with DOX, but at a ratio of 200 μg/μmol PL. For all compositions mean vesicle size was approximately 0.07 μm. Absorbance increase at 350 nm was monitored during the cooling down process (see bellow). This wavelength was chosen because of high sensitivity in detecting aggregation and little interference through DOX absorbance. The same concentration of free DOX was used as a blank. As a control, absorbance of the same liposome dispersions in the absence of DOX was also determined. Every 2–5°C, 25 μl of the incubation media were diluted to a final volume of 1 ml with 10 mM Hepes pH 7.4, 5% (w/v) glucose at the same temperature, absorbance was measured at 350 nm in a temperature-controlled Perkin Elmer λ-5 UV/VIS spectrophotometer. This dilution process was necessary in order to minimise any interference due to DOX absorbance.

2.7. Determination of DOX loading efficiency as a function of the temperature

DMPC/DPPG, DMPC/DPPG/CHOL, DPPC/DPPG, DPPC/DPPG/CHOL liposomes (mean vesicle size 0.07 μm) were incubated with DOX at a DOX/PL ratio of 25 and 100 μg/μmol for 30 min at 65°C. Subsequently, they were allowed to cool down gradually to a final temperature of 15°C. Every 5°C, 10 μl of the incubation medium were diluted to a final volume of 1 ml with 10 mM Hepes pH 7.4, 5% (w/v) glucose at the same temperature, and immediately fluorescence at excitation-emision wavelengths of 493 and 585 nm, respectively, was measured before and after disruption of the liposomes with 50 μl of 10% Triton X-100. The final DOX loading efficiency was also calculated by comparison of the fluorescence before and after removal of free DOX by treatment with Dowex as described above.

3. Results

3.1. Effect of initial vesicle size and DOX/PL ratio

Liposomes composed of DPPC/DPPG/CHOL (10:1:4) with mean vesicle size of approximately 0.07, 0.1 and 0.2 μm were prepared and loaded with different amounts of DOX by the ammonium sulfate gradient method [12]. In all cases, a tendency to an increase in vesicle size was observed upon incubation with the anthracycline (Fig. 1). This phenomenon was especially striking when the smallest liposomes (0.07 μm) were incubated with 200 μg DOX per μmol PL, the mean vesicle size being increased more than ten-fold.

3.2. Effect of lipid composition

Liposomes based on unsaturated phospholipids (EPC/EPG/CHOL, 10:1:4) were also prepared and incubated with DOX. In this case, no aggregation was observed regardless of the initial vesicle size or DOX/PL ratio, even at 24 h, 1 and 4 weeks after incubation. Moreover, during this period of time no DOX leakage was observed in any of the samples that were mantained at 4°C. The effect of the lipid composition on the aggregation of liposomes induced by DOX was further investigated. For this purpose liposomes of approximately 0.07 μm composed of DMPC/DPPG/CHOL (10:1:4) and DSPC/DPPG/CHOL (10:1:4) were prepared and incubated with different amounts of DOX. Liposomes were considered aggregated when either an increase of at least 10 nm, or a polydispersity index higher than 0.2 was measured. All freshly prepared liposome dispersions had a polydispersity index of about 0.1. Table 1 shows a correlation between the neutral phospholipid fatty acid chain length and the

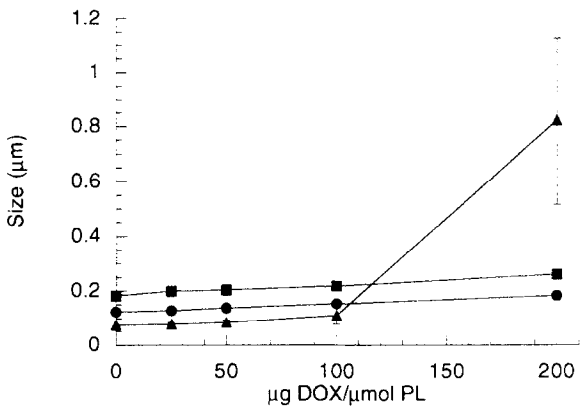

Fig. 1. The effect of the initial vesicle size and doxorubicin/phospholipid (DOX/PL) ratio on the doxorubicin-induced aggregation of liposomes composed of DPPC/DPPG/CHOL (10:1:4, molar ratio). The experiment was performed in duplicate with at least two independently prepared liposome dispersions. The initial vesicle sizes tested were 0.07 (▲), 0.1 (●) and 0.2 μm (■). Errors bars represent S.D.

Table 1
Occurrence of aggregation of liposomes upon incubation with doxorubicin and subsequent cooling as a function of the doxorubicin/phospholipid ratio (DOX/PL) and lipid composition

Ratio DOX/PL (μg/μmol)	DMPC/DPPG/CHOL	DMPC/DPPG	DPPC/DPPG/CHOL	DPPC/DPPG	DSPC/DPPG/CHOL	DSPC/DPPG
25	−	−	−	+	n.d.	n.d.
50	+	+	−	++	n.d.	n.d.
100	++	++	+	++	−	+
200	++	n.d.	++	n.d.	+	++

Incubation conditions are described in the text (Section 2). −, no aggregation; +, aggregation to a final size up to maximaly 0.2 μm; ++, final size larger than 0.2 μm.
For every lipid composition, the experiment has been performed with at least two independently prepared liposome dispersions, which have been incubated with doxorubicin at least in triplicate.

tendency of liposomes to aggregate in the presence of DOX. The shorter the chain of the PC component, the more pronounced the size increase at a certain DOX/PL ratio. The initial size of DSPC/DPPG/CHOL containing liposomes was only slightly modified after incubation with 200 μg of DOX per μmol phospholipid, whereas at the same drug/PL ratio liposomes composed of DPPC/DPPG/CHOL grew up to 1 μm. DMPC/DPPG/CHOL liposomes, on the other hand, only needed half the amount of DOX to undergo a clear increase in size. Furthermore, a protective effect of cholesterol towards aggregation was observed, since as a general trend in the absence of this molecule aggregation took place at a lower DOX/PL ratio.

All lipid compositions assayed so far had a net negative charge induced by either EPG or DPPG. In order to gain an insight into the role of the negative charge in the aggregation process, neutral liposomes (DPPC/CHOL, 10:4) as well as liposomes containing negatively charged phospholipids other than DPPG, were incubated with DOX at various drug/PL ratios (Table 2). Neutral liposomes did not undergo aggregation under any of the conditions used in this study. This suggests that the negative charge plays an important role in the aggregation process. However, other negatively charged liposomes such as those composed of DPPC/DPPS/CHOL (10:1:4) did not aggregate even when incubated with 200 μg DOX per μmol PL. Since cholesterol has proven to have a protective effect against aggregation, DPPC/DPPS containing liposomes were also included in this study. In that case, only upon incubation with 200 μg DOX/μmol PL a minor size increase was measured. When DSPG was used instead of DPPG, liposomes clearly aggregated even at a ratio of 50 μg DOX/μmol PL. Thus, a negatively charged bilayer as such does not cause aggregation. The nature of the negative charge inducer (phosphatidylglycerol) is a critical factor.

3.3. Aggregation as a function of the temperature

As a general trend, in all experiments it was observed that when aggregation occurred, it always took place during the cooling down process, after incubation of liposomes with DOX at an elevated temperature. To gain more information about the starting point of the aggregation process, liposomes composed of DMPC/DPPG, DPPC/DPPG and DSPC/DPPG were incubated with DOX at around 65°C and gradually cooled down to room temperature. Absorbance of the samples at 350 nm was monitored at different time points (Fig. 2). A similarity was found between the gel to liquid crystalline phase transition temperature of the lipid mixture (T_m) and the temperature at which a significant increase of absorbance was observed during cooling down. For DMPC/DPPG samples turbidity began to rise at 24–26°C, whereas the same phenomenon was observed at 38–41°C for DPPC/DPPG and at 50–51°C for DSPC/DPPG liposomes. T_m measured by differential scanning calorimetry (DSC) was 24.8 ± 0.1°C, 39.6 ± 0.1°C and 51.2 ± 0.0°C for DMPC/DPPG, DPPC/DPPG and DSPC/DPPG liposomes, respectively. The same experiment was also carried out for cholesterol-containing liposomes. In this case, a slight shift in the temperature of onset of aggregation was observed (31–35°C for DMPC/DPPG/CHOL and 44–47°C for DPPC/DPPG/CHOL liposomes). DSPC/DPPG/CHOL liposomes were not included in this experiment since they hardly aggregated even at a high DOX/PL ratio. In spite of the fact that no phase transition could be detected by DSC, cholesterol containing liposomes also started to aggregate at a temperature close to the respective T_m (for cholesterol-free bilayers) mentioned before.

3.4. DOX loading efficiency

Loading efficiency of all liposome preparations used in this study was determined. Almost 100% of the drug present in the incubation media associated with EPC/

Table 2
The effect of the negative charge and nature of the negative charge inducer on the aggregation of liposomes upon incubation with doxorubicin

Ratio DOX/PL (μg/μmol)	DPPC/X/CHOL				DPPC/X	
	DPPG	DSPG	DPPS	–	DPPG	DPPS
50	–	++	n.d.	n.d.	++	n.d.
100	+	++	–	–	++	–
200	++	++	–	–	n.d.	+

–, no aggregation; +, aggregation to a final size up to maximally 0.2 μm; ++, final size larger than 0.2 μm. For every lipid composition, the experiment has been perfomed with at least two independently prepared liposome dispersions, which have been incubated with doxorubicin at least in triplicate.

EPG/CHOL and about 90% with DPPC/DPPG/CHOL liposomes, regardless of the vesicle size (0.07, 0.1 or 0.2 μm) and DOX/PL ratio (25–200 μg/μmol). Only in the case of 0.07 μm liposomes composed of saturated phospholipids and incubated with 200 μg DOX/μmol PL, loading efficiency decreased below 75% (Table 3). It is noteworthy that under those conditions liposomes clearly aggregated.

With regard to the other liposome compositions, Table 3 shows that the absence of cholesterol induced a drop in the percentage of DOX that is loaded into the liposomes. This especially applies to DMPC and DPPC containing liposomes, where the loading capacity dramatically decreased in the absence of cholesterol. Moreover, a direct relationship can be observed between the chain length of the PC component and their loading capacity (DSPC > DPPC > DMPC). The amount of DOX present in the incubation media per μmol PL also proved to influence the percentage drug entrapment. In general, the higher the DOX/PL ratio, the lower the DOX loading efficiency. These observations suggest that the aggregation process is related to the drug loading efficiency, since a correlation can be established between aggregated samples and low DOX entrapment. For instance, DPPC/DPPG/CHOL liposomes, that only underwent a slight aggregation upon incubation with 100 μg DOX/μmol PL, showed a loading capacity of approximately 80%. DSPC/DPPG/CHOL liposomes, which did not aggregate under those conditions were able to entrap up to about 95% of the DOX present in the incubation media. But, DMPC/DPPG/CHOL liposomes, that clearly aggregated, only incorporated 65% of the available drug. If loading for the same lipid bilayer composition at different DOX/PL ratios is compared, the efficiency drops from 80 to 70% for DPPC/DPPG/CHOL liposomes upon incubation with 200 instead of 100 μg DOX per μmol PL. This phenomenon is more pronounced in the case of DSPC/DPPG liposomes—from 80 down to 45%. However, DSPC/DPPG/CHOL liposomes that hardly aggregated even upon incubation with 200 μg DOX/μmol PL were able to entrap up to 90% of the DOX present in the incubation media.

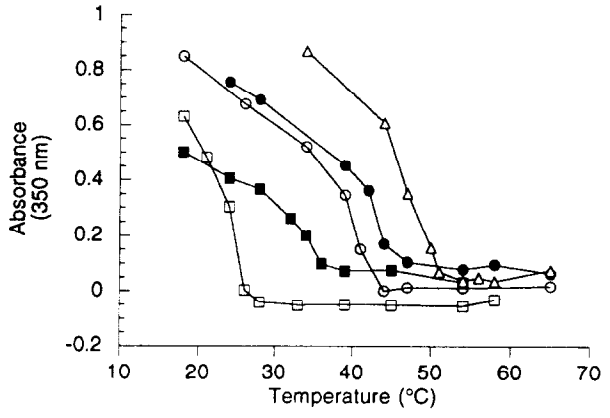

Fig. 2. Doxorubicin-induced absorbance increase of different liposome dispersions at 350 nm as a function of the temperature. The experiment was performed in triplicate with at least two independently prepared liposome dispersions for each lipid composition: DMPC/DPPG (10:1) (□), DMPC/DPPG/CHOL (10:1:4:) (■), DPPC/DPPG (10:1) (○), DPPC/DPPG/CHOL (10:1:4) (●) and DSPC/DPPG (10:1) (△). Each curve represents one independent experiment.

3.5. Effect of free DOX

To study the role of free DOX in the aggregation process, DMPC/DPPG/CHOL liposomes (0.07 μm) were incubated with 50, 100 and 200 μg DOX/μmol PL. Once samples were cooled down to about 15°C, mean vesicle size was determined before (total fractions) and after (liposomal fractions) removal of external DOX by treatment with Dowex 50W-X4. Fig. 3 shows that aggregation is partially reversible by removal of free DOX, since for the three DOX/PL ratios tested, a significant decrease in size as well as in the polydispersity index took place when the excess of free drug was eliminated. Similar results were obtained with DPPC/DPPG/CHOL liposomes (data not shown). This finding strongly supports our first impression that a certain amount of extraliposomal DOX can induce aggregation.

To further investigate to what extent extraliposomal DOX was responsible for the occurrence of aggrega-

Table 3
Doxorubicin loading efficiency (%) of liposomes (average size 0.07 μm) as a function of the lipid composition.

Ratio DOX/PL (μg/μmol)	DMPC/DPPG/CHOL	DMPC/DPPG	DPPC/DPPG/CHOL	DPPC/DPPG	DSPC/DPPG/CHOL	DSPC/DPPG
25	72 ± 5	36 ± 3	94 ± 2	44 ± 6	n.d.	n.d.
50	80 ± 7	19 ± 5	93 ± 4	14 ± 1	n.d.	n.d.
100	66 ± 7	12 ± 5	82 ± 6	24 ± 13	93 ± 5	78 ± 2
200	40 ± 2	n.d.	71 ± 8	n.d.	87 ± 10	46 ± 13

Each value represents the mean ± S.D. of two determinations with at least two independently prepared liposome dispersions.

tion, liposomes composed of either DPPC/DPPG/CHOL or EPC/EPG/CHOL and with a mean size of 0.07 μm were prepared without an ammonium sulfate gradient. Then, they were incubated for 30 min with different concentrations of DOX at 60 and 40°C, respectively, and tested for aggregation. As the driving force to entrap DOX into the liposomes (ammonium sulfate gradient) was absent, for both compositions about 15% of the drug was loaded into the liposomes. In that situation, only 20 μg DOX per μmol PL were necessary to induce aggregation of DPPC/DPPG/CHOL liposomes (Fig. 4).

The temperature at which aggregation took place was also determined by monitoring the absorbance at 350 nm from 60 down to 20°C after incubation with DOX. The temperature of onset of aggregation for these DPPC/DPPG/CHOL liposomes was independent of the presence of the ammonium sulfate gradient (around 40°C). No aggregation was observed in EPC/EPG/CHOL liposome dispersions.

3.6. DOX loading efficiency as a function of the temperature

For all the preparations tested (DMPC/DPPG, DMPC/DPPG/CHOL, DPPC/DPPG and DPPC/DPPG/CHOL), hardly any variation in the percentage of entrapped DOX as a function of the temperature was observed during the cooling down process subsequent to the DOX loading.

4. Discussion

The results of this investigation indicate that the antineoplastic agent DOX is capable of inducing aggregation of liposomes and that in this process a number of parameters are involved.

The presence of extraliposomal DOX appears to play a crucial role in the aggregation process, since in this study low loading efficiency has paralleled the occurrence of aggregation. From this observation the question arose: (1) whether low loading efficiencies during incubation at elevated temperatures were the major reason for aggregation; or (2) whether aggregation may occur first, resulting in membrane disruption and leakage of part of the liposome content.

In order to investigate the relative contribution of these two possibilities, a number of experiments were performed. Firstly, liposomes composed of DMPC/

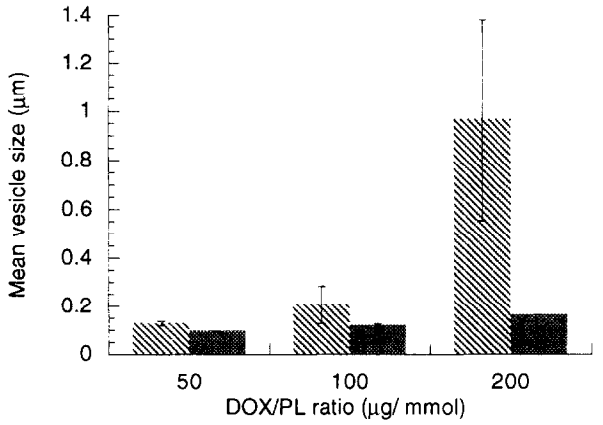

Fig. 3. Mean vesicle size of liposomes composed of DMPC/DPPG/CHOL (10:1:4) immediately after loading with doxorubicin, with (■) or without (□) removal of external doxorubicin. The initial vesicle size was 0.07 μm. Each bar represents the average of three determinations. Error bars represent S.D.

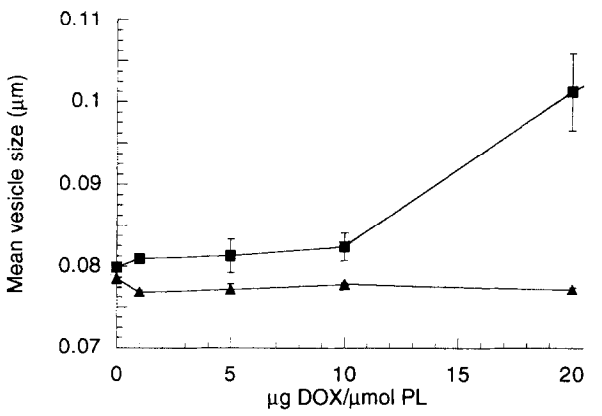

Fig. 4. Effect of free doxorubicin on the mean vesicle size of EPC/EPG/CHOL (10:1:4) (▲) and DPPC/DPPG/CHOL (10:1:4) (■) liposomes in the absence of ammonium sulfate gradient. Each value represents the average of three determinations. Error bars represent S.D.

DPPG/CHOL (0.07 μm) were prepared and incubated with 50, 100 and 200 μg DOX per μmol PL. Loading efficiency for this composition ranged from 72 to 40% depending on the DOX/PL ratio. The effect on aggregation of the DOX fraction that remained outside the vesicles was investigated by measuring the vesicle size distribution before and after removal of unentrapped DOX. In all cases aggregation took place. However, a decrease in the mean vesicle size was observed after treatment of the liposome dispersion with Dowex. This clearly indicates that extraliposomal DOX plays an important role in the increase of liposome size observed upon incubation with the drug. Furthermore, the fact that this increase in size is partially reversible suggests that particle size growth is, at least in an early stage, mostly due to aggregation and not to fusion. Secondly, DPPC/DPPG/CHOL liposomes without an ammonium sulfate gradient were prepared and incubated with the anthracycline. In this case, where low loading efficiencies were observed (15%), liposomes aggregated upon incubation with DOX at a ratio as low as 20 μg DOX/μmol PL. For the same lipid composition, but in the presence of an ammonium sulfate gradient, 100 μg DOX/μmol PL were needed to induce liposome aggregation. On the basis of the loading efficiency data (82%, for this example), one may note that also in this case the external DOX/PL ratio was around 20 μg/μmol. Finally, DOX loading efficiency was monitored after incubation of the liposomes with the anthracycline, as a function of the temperature. For all preparations hardly any variation in the percentage of entrapped DOX was observed all over the cooling down process, thus further discarding the second possibility mentioned before (aggregation followed by release).

On the basis of these results it can be concluded that the concentration of extraliposomal DOX is clearly a critical factor in the aggregation process. However, other circumstances have to coexist for aggregation to occur. A small initial vesicle size is one of those prerequisites. Liposomes larger than 0.1 μm were only slightly modified upon incubation with doxorubicin at a DOX/PL ratio as high as 200 μg/μmol, whereas under the same conditions 0.07 μm liposomes with equal bilayer composition became clearly aggregated. Similarly, small unilamellar liposomes have been reported to be more susceptible to fusion than large unilamellar vesicles [15,16], probably due to the higher degree of curvature of the former, which would render them thermodynamically less stable.

Liposome composition also proved to be important. Liposomes in the fluid state did not aggregate under any of the conditions studied. In the gel state aggregation could occur. The addition of cholesterol, known to interfere with the gel state status of the bilayer, enhanced the resistance against aggregation. Eklund et al. [17] also reported a lower threshold cation (Na^+ or Ca^{2+}) concentration for fully saturated vesicles compared to that of vesicles consisting of unsaturated phospholipids. Furthermore, for liposomes composed of DPPS or DMPG, they also observed an attenuation of vesicle aggregation in the presence of cholesterol. It is noteworthy that for all lipid compositions aggregation started above a particular DOX/PL ratio, which is in line with our previous statement that a certain amount of free doxorubicin is necessary to induce aggregation.

An interesting observation is that liposome aggregation always occurred at a fixed temperature, which differed for the various lipid compositions tested. This aggregation onset temperature is very close to the respective gel to liquid crystalline phase transition temperature (T_m) of the cholesterol-free liposomes. Similarly, although the actual figures did not match as precisely, a linear relationship has been described between the Ca^{2+}-induced fusion peak temperature of liposomes composed of bovine brain phosphatidylserine (PS), dimiristoylphosphatidylserine (DMPS) and hydrogenated bovine brain phosphatidylserine (HPS) and their phase transition temperature [18].

Quite surprisingly, even when cholesterol was present aggregation occurred at a similar temperature. It is well known that the presence of cholesterol alters the T_m of the lipid mixture. This change can range from an increase of several degrees to a total elimination of the phase transition, depending on cholesterol concentration. In all our preparations containing cholesterol (27 mol%), no phase transition could be observed by differential scanning calorimetry (DSC). However, aggregation still occurred at close proximity of the T_m found for the respective cholesterol-devoid preparations. This fact suggests that even in the presence of cholesterol some changes take place around a phospholipid dependent temperature that can not be detected by DSC. To verify whether these changes only took place in the presence of doxorubicin, the thermotrophic behaviour of DOX-containing liposomes (composed of DMPC/DPPG (10:1), DMPC/DPPG/CHOL (10:1:4), DPPC/DPPG (10:1), DPPC/DPPG/CHOL (10:1:4), DSPC/DPPG (10:1) and DSPC/DPPG/CHOL (10:1:4)) was also examined by DSC. Regardless of the absence or presence of cholesterol, no significant differences induced by doxorubicin could be observed in the thermotropic behaviour of these liposomes. Phase separation of the different liposome components, often induced by the addition of cations to membranes containing acidic and neutral lipids, could be discarded in this case. In conclusion, if changes take place in the lipid bilayer during the doxorubicin-induced aggregation process, they can not be detected by differential scanning calorimetry. Similarly, Tomita et al. [19] reported that the occurrence of interactions between doxorubicin and the membrane of temperature-sensitive liposomes was not reflected in changes in the thermotrophic behaviour of the membrane.

Nevertheless, it is well known that doxorubicin interacts with membrane structures containing anionic lipids. Whereas some authors found similar binding affinity of doxorubicin for several anionic lipids such as cardiolipin, dioleoylphosphatidic acid (DOPA), dioleoylphosphatidylserine (DOPS), and dioleoylphosphatidylglycerol (DOPG) [20], others reported a much higher affinity for cardiolipin than for phosphatidylserine [21,22]. In this study we have investigated whether the nature of the negative charge is involved in the aggregation induced by doxorubicin. We found a different behaviour in terms of inducing aggregation for the two different anionic phospholipids used. A much stronger interaction was detected for DPPG-containing liposomes than for DPPS liposomes. This is in contrast with previously published data [17], which showed that the NaCl- and CaCl-induced aggregation of both saturated and unsaturated PS vesicles occurred in the presence of lower cation concentrations compared to PG vesicles. To explain this discrepancy a higher affinity of doxorubicin for PG moiety, compared to PS, might be proposed. The fact that neutral liposomes did not aggregate under the conditions used in this study indicates that the interaction between doxorubicin and the lipid bilayer, which results in aggregation, contains a major electrostatic component. However, contributions by other interactions such as hydrophobic drug-lipid, or mutual drug-drug (stacking) interactions should not be discarded. In fact, it has been reported that doxorubicin dimers and oligomers occur in solution above 10–50 μM [20]. Extraliposomal doxorubicin concentrations in all aggregated samples clearly exceed this concentration. Therefore, it may be hypothesised that, as reported by Wolf et al. [20], free drug molecules in solution become associated by stacking interactions to the chromophore moieties of (electrostatically) bound molecules. If doxorubicin would be attached to the negatively charged bilayer through an electrostatic interaction via the posive charge of the aminosugar moiety, stacking of the chromophore moieties exposed to the aqueous medium on two colliding vesicles would result in liposome aggregation. This explanation is in line with the fact that removal of non-encapsulated doxorubicin by interaction with the cation exchange resin Dowex 50W-X4 results in the partial reversal of the aggregation state.

To conclude, the present results show that a low efficiency in loading doxorubicin into small, DPPG-containing liposomes may result in vesicle aggregation when the lipid bilayer reaches a more ordered state ($\sim T_m$). This increase in size is partially reversible by removal of non-entrapped doxorubicin, which suggests that extraliposomal doxorubicin interacts with several vesicles, thus inducing aggregation.

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References

[1] Booser DJ, Hortobagyi GN. Anthracycline antibiotics in cancer therapy: Focus on drug resistance. Drugs 1994;47:223–258.
[2] Senior J, Crawley JCW, Gregoriadis G. Tissue distribution of liposomes exhibiting long half-lives in the circulation after in vivo injection. Biochim. Biophys. Acta 1985;839:1–8.
[3] Huang KJ. Liposome pharmacokinetics. In: Ostro MJ, editor. Liposomes from biophysics to therapeutics. New York: Marcel Dekker, 1987:109–156.
[4] Gabizon A, Papahadjopoulos D. Liposome formulations with prolonged circulation time in blood and enhanced uptake by tumors. Proc. Natl. Acad. Sci. USA 1988;85:6949–6953.
[5] Storm G, Nässander UK, Vingerhoeds MH, Steerenberg PA, Crommelin DJA. Antibody-targeted liposomes to deliver doxorubicin to ovarian cancer cells. J. Lip. Res. 1994;4:641–666.
[6] Balazsovits JAE, Mayer LD, Bally MDB, Cullis PR, McDonell M, Gingsberg RS, Falk RE. Analysis of the effect of liposome encapsulation on vesicant properties, acute and cardiac toxicities, and antitumor efficacy of doxorubicin. Cancer Chemother. Pharmacol. 1989;23:81–86.
[7] Fan D, Bucana CD, O'Brian CA, Zwelling LA, Seid C, Fidler IJJ. Enhancement of murine tumor cell sensitivity to adriamycin by presentation of the drug in the phosphatidylcholine-phosphatidylserine liposomes. Cancer Res. 1990;50:3619–3626.
[8] Mayhew EG, Lasic D, Babbar S, Martin FJ. Pharmacokinetics and antitumor activity of epirubicin encapsulated in long-circulating liposomes incorporating a polyehtylene glycol-derivatized phospholipid. Int. J. Cancer 1992;51:302–309.
[9] Hwang SK, Lee KD, Hong K, Friend DS, Papahadjopoulos D. Microscopic localization of sterically stabilized liposomes in colon carcinoma-bearing mice. Cancer Res. 1992;52:5135–5143.
[10] Liu D, Mori A, Huang L. Role of the liposome size and RES blockade in controlling biodistribution and tumor uptake of GM1-containing liposomes. Biochim. Biophys. Acta 1992;1104:95–101.
[11] Mayer DL, Bally MB, Cullis PR. Strategies for optimizing liposomal doxorubicin. J. Liposome Res 1990;1:463–480.
[12] Haran G, Cohen R, Bar LK, Barenholz Y. Transmembrane ammonium sulfate gradients in liposomes produce efficient and stable entrapment of amphipathic weak bases. Biochim. Biophys. Acta 1993;1115:201–215.
[13] Rouser G, Fluscher S, Yamamoto A. Two dimensional thin layer chromatographic separation of polar lipids and determination of phopholipids by phosphorus analysis of spots. Lipids 1970;5:494–496.
[14] Storm G, Bloois L, Brouwer M, Crommelin DJA. The interaction of cytostatic drugs with adsorbents in aqueous media. The potential implications for liposome preparation. Biochim. Biophys. Acta, 1985;818:343–351.
[15] Nir S, Wilschut J, Bentz J. The rate of fusion of phospholipid vesicles and the role of bilayer curvature. Biochim. Biophys. Acta 1982;688:275–278.
[16] Boni LT, Hah JS, Hui SW, Mukherjee P, Ho JT, Jung CY. Aggregation and fusion of unilamellar vesicles by poly(ethyleneglycol). Biochim. Biophys. Acta 1984;775:409–418.

[17] Eklund KK, Takkunen JE, Kinnunen PKJ. Cation-induced aggregation of acidic phopholipid vesicles: the role of fatty acid unsaturation and cholesterol. Chem. Phys. Lipids 1991;57:59–66.

[18] Leung SO, Ho JT. Effect of membrane phase transition on long-time calcium-induced fusion of phophatidylserine vesicles. Chem. Phys. Lipids 1991;57:103–107.

[19] Tomita T, Watanabe M, Takahashi T, Kumai K, Tadakuma T, Yasuda T. Temperature-sensitive liposomes release of adryamicin, an amphipilic antitumor agent, from dipalmitoylphosphatidylcholine-cholesterol liposomes. Biochim. Biophys. Acta 1989;978:185–190.

[20] Wolf FA, Maliepaard M, Dorsten F, Berghuis I, Nicolay K, Kruijff B. Comparable interaction of doxorubicin with various acidic phopholipids results in changes of lipid order and dynamics. Biochim. Biophys. Acta 1991;1096:67–80.

[21] Goormaghtigh E, Chatelain P, Caspers J, Ruysschaert JM. Evidence of specific complex between adriamycin and negatively charged phospholipids. Biochim. Biophys. Acta 1980;597:1–14.

[22] Nicolay K, Neut R, Fok JJ, Kruijff B. Effects of adryamicin on lipid polymorphism in cardiolipin-containing model and mitochondrial membranes. Biochim. Biophys. Acta 1985;819:55–65.